OXGR1 overexpression as putative prostate cancer promoting factor

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PRO GRADU –TUTKIELMA

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Tekijä:	JÄRVINEN, SAMPSA VEIKKO SAMELI
Otsikko:	*OXGR1* geenin yli-ilmentyminen mahdollisena eturauhassyöpää edistävänä tekijänä
Sivumäärä:	55 sivua, 6 hakemistosivua
Ohjaaja:	Dosentti Leena Latonen
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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Eturauhassyöpä on yleisin miehillä diagnosoitu syöpä. Vaikka syöpä on tavallisesti hitaasti etenevä ja diagnosoidut potilaat menehtyvät tyypillisesti muun sairauden seurauksena, on eturauhassyöpä silti kolmanneksi yleisin syöpäkuolemien aiheuttaja miehillä. Nykyiset diagnostiset menetelmät eivät luotettavasti erota aggressiivisia syöpätapauksia hitaasti etenevistä. Tarvitaankin uusia taudinkulun paremmin määritteleviä menetelmiä, jotta hoitomuodot voidaan valita histopatologisten löydösten ja syövän molekulaarisien ominaisuuksien perusteella parhaan hoitotuloksen takaamiseksi. Tämän saavuttamiseksi eturauhassyövän molekulaariset mekanismit on tunnettava paremmin. Kliinisten näytteiden geenienekspressioita tutkimalla on havaittu, että *OXGR1*-yliekspressiolla saattaisi olla merkitystä joidenkin eturauhassyöpätyyppien synnyssä. Tämän tutkimuksen tarkoituksena oli selvittää *OXGR1*-yliekspression vaikutuksia kahden eturauhassyöpäsolulinjan kasvunopeuteen vaihtelevissa substraattipitoisuuksissa.

Tutkimusmenetelmät: Tutkimuksessa käytettiin aiemmin luotuja stabiilisti *OXGR1* yliekspressoivia PC-3 ja LNCaP -eturauhassyöpäsolulinjoja. Solujen ekspressioprofiilit määritettiin RT-qPCR-, western blot- ja immunofluoresenssivärjäysmenetelmillä. Kasvunopeuskokeet suoritettiin kuoppalevyillä käyttäen kasvatusliuoksessa eri pitoisuuksia glukoosia, glutamiinia ja alfaketoglutaraattia, joka on OXGR1:n ligandi. Solujen kasvu kuvattiin ja kasvunopeus määritettiin mittaamalla solujen pinta-ala ohjelmallisesti.

Tulokset: Kaikkien transfektoitujen solulinjojen *OXGR1*-mRNA -tasot olivat korkeita verrattuna kontrolleihin. Western blot -kokeet osoittivat transfektoitujen LNCaP-linjojen proteiiniekspression selvästi kasvaneen. Näiden linjojen kasvu myös nopeutui kokeessa, jossa alfaketoglutaraatin pitoisuutta lisättiin. PC-3-linjoilla proteiiniekspressio oli samankaltainen transfektoiduilla ja kontrolleilla. Kasvunopeudessakaan ei havaittu muutoksia. Western blot-kokeissa OXGR1 vasta-aineen osoittama proteiini oli yli 10 kilodaltonia odotettua suurempi.

Johtopäätökset: Tulokset antavat viitteitä, että *OXGR1*:n yliekspressiolla voisi olla merkitystä eturauhassyövän synnyssä ja osoittavat, että stabiilisti *OXGR1*:tä yliekspressoivia LNCaP -solulinjoja voidaan käyttää jatkotutkimuksissa. OXGR1 vasta-aineen oikea sitoutuminen tulee validoida ja selvittää syy havaittuun eroon proteiinin koossa. Jatkotutkimuksissa tulisi selvittää laajemmin OXGR1:n vuorovaikutuksia syövän synnyssä. Tämä olisi mahdollista toteuttaa *OXGR1*-yliekspressiolla eturauhassyövän hiirimallissa.

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ABSTRACT

Background and aims: Prostate cancer is the most common cancer diagnosed in men. While it often has indolent course, and diagnosed men more commonly perish to comorbidities, it remains the third-leading cause of cancer death in men. Current diagnostic tools fail to reliably distinguish aggressive cancer cases from indolent ones and new tools with better prognostic value are needed to address this. Preferably, a well-informed treatment choice based on histopathological findings and molecular characteristics of cancer could be made to achieve best possible treatment outcome. To succeed in this, molecular mechanisms of prostate cancer must be better understood. Based on gene expression profiles from clinical samples, overexpression of *OXGR1* could be a cancer promoting factor in certain prostate cancer subtypes. The aim of this study was to characterize effects of *OXGR1* overexpression to growth rate of two prostate cancer cell lines in varying key substrate concentrations.

Methods: *OXGR1* overexpressing PC-3 and LNCaP prostate cancer cells lines were created previously by stable transfections with pcDNA3.1 vectors having *OXGR1* under constitutive promoter. Expression profiles of cells were characterized with RT-qPCR, western blot and immunofluorescence staining. Growth rate experiments were performed in well plates using different concentrations of glucose, glutamine and alpha-ketoglutarate, which is a ligand for OXGR1, in growth medium. Cells were imaged at intervals and growth rate was defined by measuring surface area programmatically.

Results: All transfected cell lines had high *OXGR1* mRNA levels compared to controls. In LNCaP cell lines western blot results showed clear increase in protein expression of transfected lines. In transfected LNCaP cell lines an increase in growth rate was seen as the alpha-ketoglutarate concentration was increased. In PC-3 cell lines the protein levels were similarly low regardless of transfection status. *OXGR1* overexpression had no effect on growth rate of PC-3 cells in any of the growth conditions. OXGR1 antibody used in western blot experiments produced a clear band over 10 kilodaltons larger than the predicted band size.

Conclusions: These results show promise that *OXGR1* overexpression could have significance in prostate cancer promotion and that stable *OXGR1* expressing LNCaP cell lines can be used to study this further. However, proper binding of the OXGR1 antibody must be validated and the cause of shift in band size elucidated. Studies that take more molecular interactions into account are probably needed to clarify the role of *OXGR1* overexpression in prostate cancer. This could possibly be done by performing *OXGR1* overexpression studies in prostate cancer mouse model.

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Tampere, November 2017

Sampsa Järvinen

ABBREVIATIONS

ADT	Androgen deprivation therapy
AKG	Alpha-ketoglutarate
AR	Androgen receptor
AR-V7	Androgen receptor splice variant 7 mRNA
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CNA	Copy-number aberration
CRPC	Castration resistant prostate cancer
CTC	Circulating tumor cell
DRE	Digital rectal examination
DTT	Dithiothreitol
EBRT	External beam radiotherapy
FDA	Food and Drug Administration
hK2	Human kallikrein 2
HRP	Horseradish peroxidase
LHRH	Luteinizing hormone-releasing hormone
lncRNA	Long noncoding RNA
LOH	Loss of heterozygosity
LTE_4	Leukotriene E_4
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
mCRPC	Metastatic castration resistant prostate cancer
MRI	Magnetic resonance imaging
OXGR1	2-Oxoglutarate receptor 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA3	Prostate cancer antigen 3
PCGEM1	Prostate cancer gene expression marker 1
PHI	Prostate health index
PMSF	Phenylmethylsulfonyl fluoride
PSA	Prostate-specific antigen
SRE	Skeletal-related events
TBP	TATA-binding protein

CONTENTS

1 INTRODUCTION

Prostate cancer is the most common cancer in males in Finland and the second most common cancer in males worldwide (Ferlay et al. 2015; Finnish Cancer Registry 2017). Although prostate cancer often has indolent course, and is typically a disease of elderly men, it remains the second leading cause of cancer-related death in Finland and a cause for distress for the families affected (Finnish Cancer Registry 2017). Most important risk factors for prostate cancer are age, African-American ethnicity, and family history. One third of the familiar increase in risk of being diagnosed with prostate cancer is accounted for by known prostate cancer associated genetic aberrations (Olama et al. 2014). Several protective factors, such as physical activity, vitamin intake, diet, and various drugs have been studied, but nothing with substantial benefits have been found.

Almost all prostate cancers are adenocarcinomas that develop when glandular prostate cells become malignant. These cells grow into localized tumors that can either be contained in the prostate, or spread to surrounding tissues such as seminal vesicles, bladder, or the rectum (Prostate cancer: Current Care Guidelines, 2014). Prostate cancer most commonly metastasizes to the bones and lymph nodes. Localized cancer can be curatively treated by either radical prostatectomy or radiotherapy, though a fraction of cases develops into recurrent metastatic disease. Metastatic disease is treated with androgen deprivation therapy (ADT), which halts the disease progression until castration resistant prostate cancer (CRPC) develops. CRPC is always fatal, but can be treated with new drugs that interfere with androgen pathway and slow disease progression. Heterogenous nature of prostate cancer has made characterization of mechanisms of disease initiation and progression difficult. Recent advances in high-throughput genetic technologies together with mechanistic studies are providing important information, but translation of the data to advances in treatment and diagnostics is still in infancy.

Prostate cancer diagnosis is made based on an ultrasound biopsy, which is taken if digital rectal examination (DRE) and prostate-specific antigen (PSA) levels in blood give reason to suspect cancer (Mohler et al. 2016; Prostate cancer: Current Care Guidelines, 2014). PSA is not specific to prostate cancer and is increased also in benign conditions of prostate. Indolent cancer cases cannot reliably be discriminated from aggressive ones based on biopsy and PSA levels alone. These are the main reasons that have led to overdiagnosis and overtreatment of prostate cancer, which, as widespread consensus acknowledges, are problems that should be addressed by more accurate diagnostic and prognostic tools.

In the literature review section of this thesis, an overview of current knowledge on epidemiology and risk factors, and of state of the art practices in diagnosing and treating prostate cancer is given. The main focus is on genetic aberrations underlying prostate cancer, and on emerging biomarkers that show promise in better characterization of the disease in its early stages, making an informed choice of the best treatment options possible.

In the study section, the possible role of *OXGR1* in prostate cancer is assessed. High-throughput RNA sequencing data of clinical prostate cancer samples show significant overexpression of *OXGR1* in a small subset of samples. As there is no published data suggesting *OXGR1* to be a cancer promoting factor, the main aim of the study was to investigate whether, and by which mechanisms, *OXGR1* expression and the protein coded by it, 2-Oxoglutarate receptor 1 (OXGR1), could confer growth advantage to prostate cancer cells.

2 REVIEW OF THE LITERATURE

2.1 Prostate cancer

2.1.1 Epidemiology of prostate cancer

Prostate cancer is the most common cancer in males in Finland and the second most common cancer in males worldwide (Ferlay et al. 2015; Finnish Cancer Registry 2017). Number of new diagnosed cases per year (incidence) in Finland in 2015 was 4855 and number of deaths per year in 2015 was 921, which translates to 14% of all cancer deaths in men (Finnish Cancer Registry 2017).

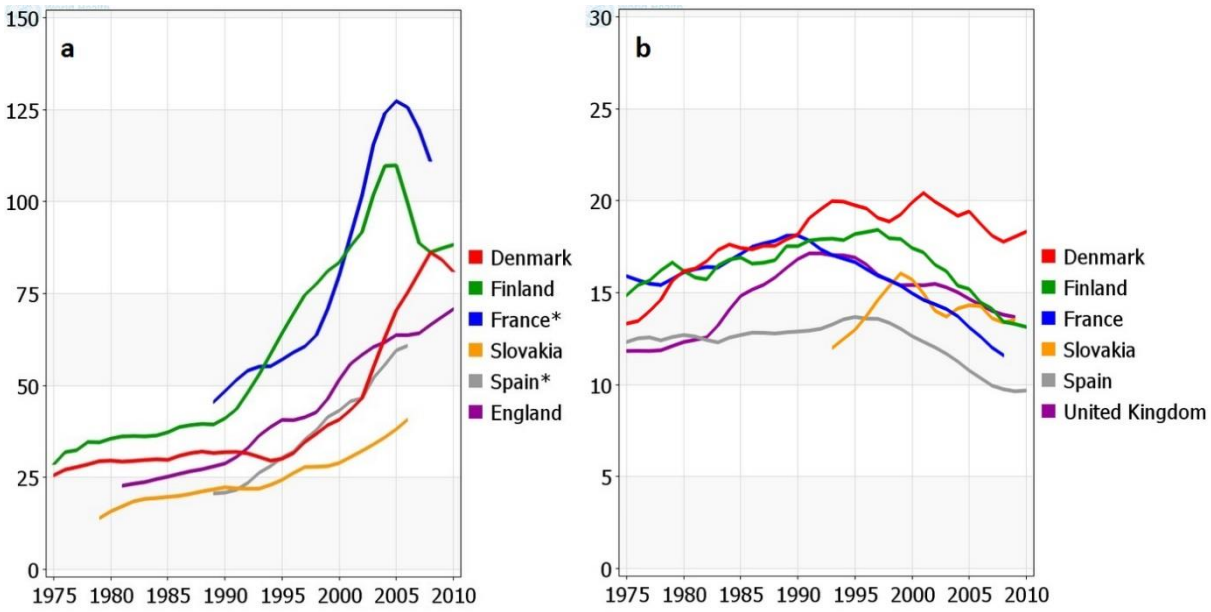

Figure 1: Incidence and mortality of prostate cancer in selected countries. a) Age-standardized incidence per 100 000. * Regional data. b) Estimated (WHO) age-standardized mortality per 100 000. WHO's world standard population was used in age standardization. Figure from Ferlay J et al, 2013.

As seen in Figure 1, the incidence of prostate cancer began to increase dramatically in the developed countries during the 1990's, while mortality has begun to decrease slightly during the same period. The increase in the incidence is mainly explained by the development and wide use of PSA testing which has led to diagnosis of docile prostate cancer cases that would have otherwise remained undiagnosed (Schroder et al. 2014). At least part of the simultaneous decrease in mortality rate may also be attributed to the advent of PSA testing (Kvale et al. 2007; Schroder et al. 2014). Other mortality decreasing factors include the adoption of early curative treatment of localized prostate cancer with radical prostatectomy or radiotherapy, and improved treatment of more advanced disease (Kvale et al. 2007).

2.1.2 Risk factors

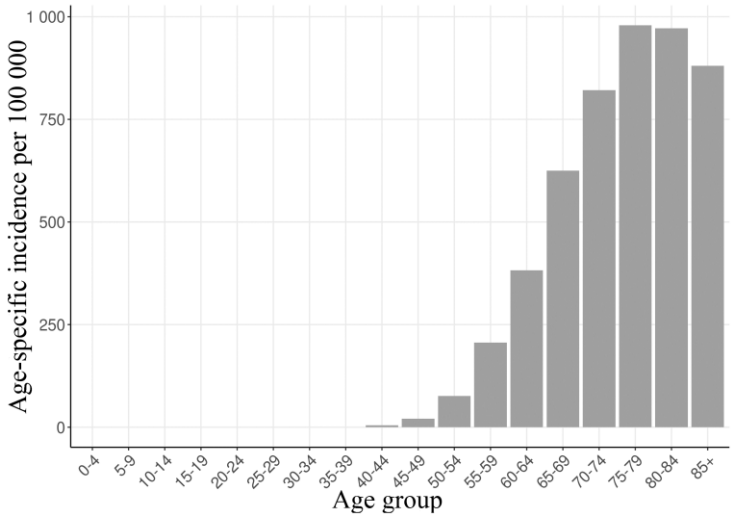

Figure 2: Age-specific incidence of prostate cancer in Finland in 2011-2015. Figure modified from Finnish Cancer Registry (https://tilastot.syoparekisteri.fi) (9.10.2017).

While the pathogenesis of prostate cancer is not completely understood, several risk factors that increase individuals' likelihood to get cancer have been established. As seen in Figure 2, the risk of being diagnosed with prostate cancer rises dramatically as the individual ages (Finnish Cancer Registry 2017). Aging increases prostate cancer risk in different ways. As with almost all cancers, aging allows for more time for accumulation of DNA damage in the prostate both due to internal and external carcinogens (Serrano and Blasco 2007). Inflammation processes have been linked to initiation and progression of prostate cancer, and immune system function is altered with aging (Sfanos and De Marzo 2012). This is characterized for example by decreased T cell differentiation and increased amount of pro-inflammatory cytokine secretion (Fagnoni et al. 2000; Kim et al. 2011). These processes have been linked to prostate tumorigenesis in prostate cancer cell line studies (De Angulo et al. 2015).

Prostate cancer incidence rates vary greatly depending of race. Age-adjusted incidence rates per 100 000 people in the United States during 2010-2014 were 189, 113, and 63, for African-American, Caucasian, and Asian men, respectively (National Cancer Institute 2017). Black men are also found to have from 44% to 75% higher risk at the time of diagnosis, compared to general population, to develop metastatic disease (Tsodikov et al. 2017). There is also geographic variation in prostate cancer incidence. For example, Asian men living in United States have higher incidence than in their country of origin (Goggins and Wong 2009). The reasons for this are unclear, though differences in screening and lifestyle changes are likely to effect (Goggins and Wong 2009; Hemminki et al. 2013).

Men with first-degree relative diagnosed with prostate cancer have 2.5 times higher risk to develop the disease (Kicinski et al. 2011). The risk is higher in men under 65 (2.9) and even higher with men whose affected relative is a brother (3.14) (Kicinski et al. 2011). 33% of the familiar risk is accounted for in the European ancestry population with most susceptibility

loci conferring only a small increase in risk (Olama et al. 2014). Men under 65 with rare *BRC1* and *BRCA2* mutations have in estimate 3.4 and 8.6 times higher risk of prostate cancer and are more likely to develop aggressive disease (Kote-Jarai et al. 2011; Leongamornlernt et al. 2012). HOXB13 G84E mutation also confers 3.4 times higher risk of developing prostate cancer, but other mutations conferring similar risk increases haven't been detected (Cuzick et al. 2014; Karlsson et al. 2014).

There is some evidence that ionizing radiation used in diagnostic procedures and UV radiation from sun exposure could increase prostate cancer risk, but the studies are limited (Myles et al. 2008; Nair-Shalliker et al. 2012). The role of urinary tract infections in development of prostate cancer has been studied, and *Trichomonas vaginalis* infection and chronic intra-prostatic inflammation might be involved in the development of prostate cancer (De Marzo et al. 2007; Sutcliffe et al. 2012).

A meta-analysis of 24 prospective cohort studies found that cigarette smoking modestly increases prostate cancer mortality, but not incidence (Islami et al. 2014). This is substantiated by a study examining biopsy-negative men with protocol-dictated PSA-independent rebiopsies that found smoking to be associated with higher grade disease, but not with overall prostate cancer diagnosis (Ho et al. 2014).

An overview of studies suggests that higher BMI is associated with an increased risk of advanced prostate cancer but a decreased risk in localized disease (Discacciati and Wolk 2014). Many dietary factors have been studied, but there is no solid evidence for association with prostate cancer (Discacciati and Wolk 2014). A large meta-analysis found that physical exercise slightly reduces the risk for prostate cancer (Liu et al. 2011).

Many protective factors, such as the higher intake of antioxidant vitamin E and selenium, cofactor of several antioxidant enzymes, have been studied extensively, but they were not found to reduce the risk of prostate cancer (Klein et al. 2011). There is limited evidence from cohort studies and randomized trials that regular aspirin intake could reduce prostate cancer incidence and mortality (Bosetti et al. 2011; Rothwell et al. 2011). However, results are heterogenous and the dose and duration-risk relationships are not clear.

2.1.3 Diagnosis

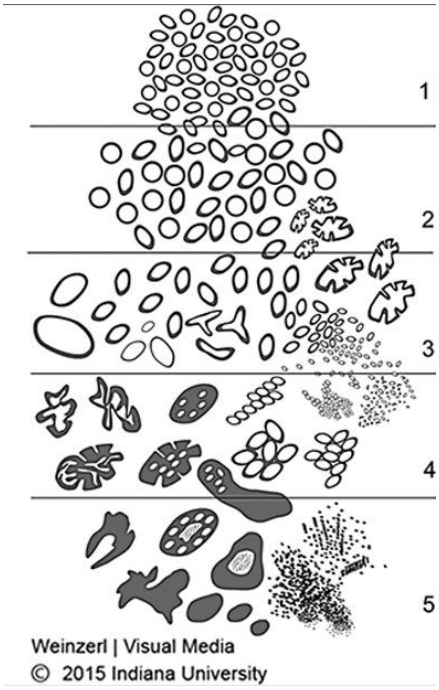

Figure 3: A Gleason grading chart. Image from Epstein et al. 2016.

If DRE and / or blood test for PSA give reason to suspect prostate cancer, a transrectal ultrasound systematic prostate biopsy of 10 to 12 tissue samples from different parts of the prostate is taken (Mohler et al. 2016; Prostate cancer: Current Care Guidelines, 2014). This biopsy is the main basis for diagnosis. A Gleason grade from 1 to 5 is issued based on gland differention in the tissue samples, with 1 being well differentiated (Figure 3). A primary Gleason grade is issued based on what pattern is most commonly seen and a secondary Gleason grade is issued based on the second most commonly seen pattern. A recently introduced and adopted system, attempting to better discriminate between indolent and aggressive cancer types, grades prostate cancer to 5 groups based on Gleason scores (table 1) (Epstein et al. 2016). If metastasis of cancer is suspected on grounds of symptoms, high grading, high PSA or other risk factors, bone scans and other imaging tests are performed (Prostate cancer: Current Care Guidelines, 2014).

Table 1: Grading of prostate cancer. Table modified from Epstein et al. 2016.

Risk grade	Gleason scores	Histological description
Grade 1 cancer	Gleason score of ≤ 6	Only individual, discrete, well-formed glands
Grade 2 cancer	Gleason score of 3 + 4	Predominantly well-formed glands with lesser component of poorly formed, fused, or cribiform glands
Grade 3 cancer	Gleason score of 4 + 3	Predominantly poorly formed, fused, or cribiform glands with lesser component of well-formed glands
Grade 4 cancer	Gleason scores of 4 + 4, 3 + 5, and 5 + 3	Only poorly formed, fused, or cribiform glands or well-formed glands plus area lacking glands
Grade 5 cancer	Gleason scores of 4 + 5, 5 + 4, and 5 + 5	Lacks gland formation (or with necrosis) with or without poorly formed, fused, or cribiform glands

21 to 28 percent of cancer cases are missed when only a systematic biopsy is used for diagnosis (Bjurlin et al. 2013). Serum PSA variant levels, along with emerging biomarkers such as *PCA3* and DNA methylation status of prostate biopsy tissue, can be utilized in search for false negatives (Gupta et al. 2010; Partin et al. 2014; Wei et al. 2014). Another recent development is the use of multiparametric magnetic resonance imaging (MRI) in performing targeted biopsies, commonly in addition to systematic biopsies (Weinreb et al. 2016; Filson et al. 2016). It holds promise of more specific and sensitive diagnosis, but further validation and setting of guidelines is still needed (Rosenkrantz et al. 2016).

2.1.4 Treatment

Localized prostate cancer: When deciding treatment strategy for localized prostate cancer, accurate prognosis, patient age and life expectancy, effects of chosen treatments side effects on the quality of life, and patient's comorbidities all play an important role (Litwin and Tan 2017). Most important prognostic factors are clinical staging, which takes into account the spreading of the disease, cancer grade (table 1), and serum PSA levels (Prostate cancer: Current Care Guidelines, 2014). Accurate prognosis, especially for cancer cases with Gleason scores 6 and 7, has proven difficult with the traditional methods (Irshad et al. 2013). New gene assays attempting to predict cancer progression from biopsy tissue samples have been presented with some success (Klein et al. 2014; Knudsen et al. 2016; Sommariva et al. 2016). A 14-year follow up study to patients with nonmetastatic prostate cancer at diagnosis found other-cause mortality rates ranging from 24% to 57%, depending from the number of co-morbidities, whereas the prostate cancer-specific mortality ranged from 3% to 18%, depending of risk rating based on clinical stage, Gleason score, and PSA level (Daskivich et al. 2013). Decision aids for prostate cancer patients have been introduced to facilitate shared decision making, which could better take into account effects to quality of life caused by the side effects of chosen treatment. A meta-analysis of decision aid trials states that only limited or no benefits have been gained by their use, so further development and study is needed (Violette et al. 2015).

Three primary treatment options are available for localized prostate cancer: Expectant management, surgery and radiation. Expectant management can be divided to watchful waiting, where disease progress is monitored by patient's symptoms only, and active surveillance, which consists of serial PSA testing, physical examinations and rebiopsies (Filson et al. 2015). This approach is taken to limit the adverse effects of over treatment and is supported by studies with selected low-risk patients with Gleason scores 7 or less undergoing active surveillance finding the risk of metastasis and prostate cancer mortality to range from 0% to 6.1% (Godtman et al.

2016; Hamdy et al. 2016; Klotz et al. 2015; Tosoian et al. 2015; Welty et al. 2015). One of these studies compared the 10 year outcomes of active surveillance, surgery and radiation therapy, finding only minor, non-significant differences in prostate cancer-specific mortality and better quality of life in men with active surveillance (Hamdy et al. 2016; Donovan et al. 2016).

Surgery or radiation therapy are chosen as treatment when the disease is deemed more likely to advance, either at primary diagnosis or after worsened prognosis during active surveillance (Litwin and Tan 2017). Commonly used indications are PSA value of over 10 ng / ml and nodules palpable on DRE (Litwin and Tan 2017). Surgery is a radical prostatectomy, where whole prostate is removed with its capsule. Also, regional lymph nodes can be removed if biopsies (Gleason score ≥ 8) and PSA value (>10 µg / l) suggest more aggressive disease (Prostate cancer: Current Care Guidelines, 2014). The use of robot-assisted laparoscopic prostatectomy has quickly become the standard procedure, and it has been shown to reduce adverse effects in comparison to open radical prostatectomy, such as loss of urinary and sexual function (Ficarra et al. 2012a, Ficarra et al. 2012b). The curative power of these surgeries is considered similar, as no significant difference in proportion of positive surgical margins has been found (Yaxley et al. 2016). Adjuvant radiotherapy is considered, and has been found effective in long term trials, after surgery in high risk patients, such as those with positive surgical margins (Bolla et al. 2012; Wiegel et al. 2014).

Radiation therapy is commonly combined with ADT (Mohler et al. 2016). 6 months of ADT in intermediate risk patients and ≥ 24 months of ADT in high risk patients, both started before radiation therapy, have been found effective in randomized clinical trials (Bolla et al. 2016; Zapatero et al. 2015). Radiation therapy is carried out as external beam radiotherapy (EBRT) or brachytherapy in case of intermediate risk or a combination of both in high risk patients (Mohler et al. 2016). Recent advances in EBRT include better radiation beam targeting, enabling higher radiation dose delivery to prostate, reduction of side effects caused by radiation to surrounding organs, and improved cancer control (Dearnaley et al. 2014; Viani et al. 2016).

Previous studies summarized in meta-analysis have indicated lower mortality with surgery than radiotherapy, however, first randomized comparison of these treatment options found no difference in prostate cancer mortality (0.9% and 0.7 for surgery and radiation, respectively) or clinical progression (8.3% and 8.4% for surgery and radiation, respectively) during 10-year follow-up (Hamdy et al. 2016; Wallis et al. 2016). After surgery, the prostate can be fully analyzed to accurately grade the disease. Also, PSA should be undetectable when no normal or cancerous prostate cells exist, enhancing monitoring for recurrence (Paller and

Antonarakis 2013). This is not the case with radiation therapy, after which the PSA values typically decrease, but fluctuation is also observed (Djavan et al. 2003). Another important consideration when choosing proper treatment is the impact of side effects to the quality of patients' life. Most important side effects are sexual dysfunction and urinary incontinence for radical prostatectomy, and sexual dysfunction and bowel problems for radiation therapy, but these are affected by baseline functions in patients (Chen et al. 2009).

Metastatic Prostate Cancer: Prostate cancer commonly metastasizes to bone (80 to 90 percent of metastatic cases) and lymph nodes (40 to 50 percent of metastatic cases) (Mohler et al. 2016). The standard therapy to metastatic disease is ADT (Prostate cancer: Current Care Guidelines, 2014). ADT is performed by orchiectomy, luteinizing hormone-releasing hormone (LHRH) agonists, or antagonists which all result in considerably lowered androgen levels (Gillessen et al. 2017). This treatment strategy halts the disease progression for ~ 0.5 to 2 years, after which a castration resistant disease is developed (James et al. 2015). Due to serious side effects of ADT, such as decreased bone mineral density, metabolic changes, sexual dysfunction, cardiac morbidity and cognitive dysfunction, the effect of intermittent ADT has been studied (Nead et al. 2017; Nguyen et al. 2015). A recent meta-analysis found intermittent ADT as effective as standard ADT, with minor improvements in physical and sexual function (Magnan et al. 2015). A well tolerated cytostatic, docetaxel, can be combined with ADT, with two randomized trials reporting considerable delays in cancer progression and increases in cancer survival times (James et al. 2016; Sweeney et al. 2015).

ADT is advised to be continued when cancer progresses to castration resistant state, although there are no recent studies confirming its usefulness with the new treatment options (Cornford et al. 2017; Hussain et al. 1994). Two new drugs targeting androgen pathways have been developed and recently adopted for treatment of metastatic castration resistant prostate cancer (mCRPC) (Cornford et al. 2017). Abiraterone acetate inhibits androgen biosynthesis and enzalutamide interferes with androgen receptor (AR) signaling. Both have been shown to slow disease progression and improve survival and quality of life in randomized clinical trials (de Bono et al. 2011; Scher et al. 2012). Docetaxel has also been found beneficial for survival times and can be used as treatment for mCRPC if not utilized before (Armstrong et al. 2010; Cornford et al. 2017). Another emerging treatment option for asymptomatic or minimally symptomatic mCRPC is Sipuleucel-T, a cancer vaccine found to increase median survival time for 4 months (Kantoff et al. 2010). Common painful bone metastases can be palliatively treated with radiation therapy, which can considerably improve quality of live (Hartsell et al. 2005). Bone targeting agents such as denosumab and zoledronic acid can be used to reduce skeletal-related

events (SRE) associated with mCRPC, such as fractures and spinal cord compression (Saad et al. 2002; Smith et al. 2012). ^{223}Radium, an α-emitter particle that selectively binds to bony metastases is the first bone-targeted therapy shown to increase prostate cancer survival. In randomized trial, it increased median overall survival by 3.6 months and delayed first SRE by 5.8 months, in comparison to placebo (Parker et al. 2013).

Quality of life considerations: From 2006 to 2012, the five-year survival rates for local and regional disease were close to 100% and for distant metastatic disease 30% (Siegel et al. 2017). In Finland, the 5-year survival for all diagnosed prostate cancer cases in 2013-2015 was 94% (Finnish Cancer Registry 2017). As prostate cancer can be better controlled with new treatment options and strategies, treatments and interventions supporting active life are becoming increasingly important. Couples therapy and treating of erectile dysfunction caused by ADT and radiotherapy with sildenafil have been found to improve quality of life (Couper et al. 2015; Watkins Bruner et al. 2011). Pelvic floor muscle exercise has been shown to improve urinary continence after localized prostate cancer treatment (Zhang et al. 2015). Diet and exercise interventions especially for patients undergoing ADT for metastatic disease have proven to be effective in improving quality of life (Bourke et al. 2014; Bourke et al. 2016).

2.1.5 Genetics of prostate cancer

In over 80% of men diagnosed with prostate cancer, multiple tumor foci are detected in the prostate (Svensson et al. 2011). Recent studies utilizing whole genome sequencing (WGS) and exome sequencing suggest that these foci are of independent origin (Cooper et al. 2015; Lindberg et al. 2013). This find is supported by a study examining the differences in the most common genetic rearrangements between different tumor foci (Svensson et al. 2011). Interestingly, analysis of WGS data from tumors and their metastatic sites reveals that metastases commonly originate from single foci (Gundem et al. 2015). After metastasis, re-seeding from metastases to primary foci and other metastases commonly takes place, reducing tumor heterogeneity (Gundem et al. 2015; Hong et al. 2015). This is especially important during chemotherapy, since there is evidence of cross-metastatic seeding of treatment resistant cells (Hong et al. 2015).

Prostate cancer is characterized by gene fusions and copy-number aberrations (CNA) with only some point mutations of general importance. This makes pinpointing the genes primarily responsible for oncogenesis difficult, since for example loss of heterozygosity (LOH) and chromosomal gains can cause differential expression of multitude of genes. Some progress

has been made by pan-cancer analyses of CNAs, suggesting for example that deregulation of DNA mismatch repair could predispose to prostate cancer (Zack et al. 2013). Some of the most important known chromosomal rearrangements and mutations, along with epigenetic features, for prostate cancer development and progression are summarized in the following section.

ETS fusion genes: ETS transcription factor gene fusions are detected in about half of prostate cancer cases. The most common of these is the linkage of *TMPRSS2* androgen-responsive promoter and the transcription factor gene *ERG* (Tomlins et al. 2007). At least one mechanism of the fusion is thought to be prolonged exposure to androgens, increased AR activity and inhibition of the double-strand break preventing protein PIWIL1 (Bastus et al. 2010). AR transcription complex brings *ERG* and *TMPRSS2* loci in contact and DNA double-strand break repair mechanisms can lead to fusion of the two genes (Mani et al. 2009; Wu et al. 2011). ETS fusion positive prostate cancer cases are often associated with additional inter-dependent chromosomal rearrangements appearing early in cancer progression (Baca et al. 2013). The precise mechanism by which these rearrangements occur is not fully known, but there is evidence of AR signaling playing a key role (Weischenfeldt et al. 2013). *TMPRSS2-ERG* gene fusions have been studied extensively, but taken alone their explanatory power to prostate cancer progression is inconsistent (Tomlins et al. 2009). However, *TMPRSS2-ERG* fusions are associated with younger age at diagnosis and lower PSA values (Schaefer et al. 2013). When observed with other defects such as increased AR activity or *PTEN* loss, *TMPRSS2-ERG* fusions are shown to lead to invasive adenocarcinoma (Carver et al. 2009).

Androgen signaling: AR is a hormone receptor that mediates its effects primarily as a DNA binding transcription factor in response to androgen stimuli (Heinlein and Chang 2002). It has also been shown to have non-genomic actions for example by directly inducing the MAPK signal cascade in the cytoplasm (Heinlein and Chang 2002). AR signaling is crucial to development of both normal prostate and its cancer and androgen ablation therapy is standard treatment for advanced metastatic prostate cancer (Shen and Abate-Shen 2010). AR signaling can be aberrantly increased and become ligand-independent, thus promoting the development of androgen-independent prostate cancer, due to *AR* gene amplification, point mutations and alternative splice variants (Grasso et al. 2012; Visakorpi et al. 1995). These are detected already in localized tumors, but much more frequently in castration resistant cases (Grasso et al. 2012). Mutations and CNAs of genes regulating AR signaling have been detected in both localized and metastasized prostate cancer cases. *FOXA1* codes for an AR cofactor and its recurrently detected mutations are shown to increase tumor growth (Grasso et al. 2012). Another such gene

is *NCOA2*, whose overexpression is shown to increase recurrence rates after radical prostatectomy (Taylor et al. 2010).

LOH in 5q and 6q regions: Losses in genetic material in 5q and 6q chromosomal arms are mainly seen in ETS fusion negative cases of prostate cancer (Mitchell and Neal 2015). The most notable prostate cancer linked gene in 5q arm is *CDH1*. In addition to LOH, *CDH1* point mutations and rearrangements have also been reported in prostate cancer (Grasso et al. 2012). Loss of *CDH1* is associated in increase in both CNAs and intra-chromosomal re-arrangements in prostate cancer (Baca et al. 2013). *CDH1* codes for cadherin protein with important role in cell adhesion. Its downregulation in prostate cancer is associated with loss of differentiation, advanced clinical stage, and poor survival (Ikonen et al. 2011). Losses of genetic material in 6q12-6q22 region are common in prostate cancer and associate with aggressive disease (Kluth et al. 2013; Williams et al. 2014). *MAP3K7* is thought to be the key tumor suppressor gene in the region, since it governs transcription regulation and apoptosis pathways (Mihaly et al. 2014).

PTEN inactivation: *PTEN* is a tumor suppressor gene that downregulates PI3K-dependant signaling. PI3K-pathways govern cell growth, proliferation, differentiation, motility and survival. *PTEN* deletions are reportedly detected in 20 to 60 percent of localized prostate cancer cases (Phin et al. 2013). *PTEN* loss is associated with ETS fusion positive cases, but is also detected in fusion negative cases, in which it seems to have the most negative effect to patient survival (Reid et al. 2010). Patients with no *PTEN* loss and no ETS rearrangements are reported to have better survival (Reid et al. 2010). PTEN activity can alternatively be diminished by aberrant expression of linked genes *MAGI2* and *PIK3CA*, which have been associated with prostate cancer (Barbieri et al. 2012; Berger et al. 2011). PTEN is also shown to be downregulator of AR activity, and loss of *PTEN* is linked to development of androgen-independent prostate cancer (Mulholland et al. 2006).

RB1* and *TP53: *RB1* and *TP53* are both important cell cycle regulators and tumor suppressor genes commonly inactivated in multitude of cancer types. Loss of *RB1* function is associated with onset of castrate-resistant prostate cancer and is detected in almost half of these cases (Grasso et al. 2012; Weischenfeldt et al. 2013). *TP53* is commonly mutated or lost in both localized and progressed cases. Recent study with prostate cancer mouse models reports that *RB1* loss facilitates metastatic processes initiated by loss of *PTEN* and that loss of *TP53* causes resistance to antiandrogen therapy (Ku et al. 2017).

***MYC*:** *MYC* is a proto-oncogene that drives cell cycle progression and cell survival processes. It is commonly amplified or overexpressed in prostate cancer (Gurel et al. 2008;

Taylor et al. 2010) and its overexpression is associated with more aggressive disease and cancer recurrence (Hawksworth et al. 2010; Rye et al. 2014).

***NKX3-1*:** LOH in *NKX3-1* is a common aberration in prostate cancer, typically resulting from deletion of chromosomal region 8p (Baca et al. 2013). *NKX3-1* codes for a putative tumor suppressor transcription factor Nkx3.1, which in mouse studies is important to normal prostate epithelial differentiation and stem cell function (Shen and Abate-Shen 2010).

***SPOP*:** *SPOP* is the gene with most frequently detected point mutations in prostate cancer and these mutations are mainly seen in ETS fusion negative cases (Barbieri et al. 2012). Protein coded by *SPOP* is a part of ubiquitin-protein ligase complex and is shown to stabilize the oncogene *DEK* by modifying its ubiquitylation state. This in turn was shown to increase invasive capabilities of prostate epithelial and LNCaP cells *in vitro* (Theurillat et al. 2014). Other possible prostate cancer-related target proteins for SPOP include ERG, Myc, and TRIM24 (Gan et al. 2015; Theurillat et al. 2014).

MicroRNAs and long noncoding RNAs in prostate cancer: Expression profiling studies of prostate cancer tissue samples show a large number of microRNAs aberrantly expressed in prostate cancer, with several of both up and downregulated microRNAs showing up in most of the material (Goto et al. 2015; Martens-Uzunova et al. 2012). A single microRNA typically regulates the expression of multiple genes and both probable tumor suppressive and oncogenic microRNAs have been identified (Fabris et al. 2016). Among the most interesting microRNAs are miR-21 and miR-141, whose overexpression has been associated with the development of castration resistant prostate cancer and metastatic disease, respectively (Ribas et al. 2009; Yaman Agaoglu et al. 2011). Interestingly, a recent study states that miR-141 has an inhibitory effect on metastasis and is actually downregulated in prostate cancer (Liu et al. 2017). MicroRNA signatures are considered promising biomarkers, since they can be measured from different biological samples, they are relatively easy to detect and quantify, and they are stable molecules (Chen et al. 2008).

Data acquired by deep sequencing of prostate cancer transcriptome shows that long noncoding RNAs **(**lncRNAs) are abundantly expressed and several prostate cancer-specific lncRNAs have been identified (Prensner et al. 2011; Walsh et al. 2014; Ylipää et al. 2015). Together with deep sequencing data, functional studies suggest several mechanisms by which lncRNAs promote carcinogenesis. Prostate cancer gene expression marker 1 (*PCGEM1*), has shown to be overexpressed in over half of prostate tumors and reported to promote cell proliferation through modification of gene expression by enhancer-promoter looping (Petrovic et al. 2004). This is a feature commonly recognized in lncRNAs (Lai et al. 2013). Metastasis-

associated lung adenocarcinoma transcript 1 (*MALAT1*) is overexpressed in prostate cancer and is associated with disease severity in other cancer types (Ji et al. 2003; Lin et al. 2007). *MALAT1* has been shown to regulate expression of several genes through modulation of alternative splicing (Bernard et al. 2010). Other cancer promoting mechanisms include antisense gene silencing and repressing of DNA repair mechanisms (Walsh et al. 2014). Prostate cancer antigen 3 (*PCA3*) is a lncRNA highly specific to prostate cancer, though not much of its function is known, and a *PCA3* test for prostate cancer diagnosis is in clinical use (Bussemakers et al. 1999; Groskopf et al. 2006). *MALAT1* has also been proposed as a prostate cancer biomarker that could be measured from blood plasma (Ren et al. 2013).

DNA methylation in prostate cancer: As with other cancers, CpG DNA methylation is highly deregulated in prostate cancer (Valdes-Mora and Clark 2015). In broad view, genomic DNA is hypomethylated, which is thought to lead to genome instability and activation of oncogenes (Brothman et al. 2005; Cho et al. 2009; Valdes-Mora and Clark 2015). Another characteristic is hypermethylation of certain promoter sequences. Over 50 genes, which are involved in cellular pathways critical for cancer development and progression such as cell cycle control, hormone response, DNA repair, tumor invasion and apoptosis, have been identified as commonly hypermethylated in prostate cancer (Jeronimo et al. 2011).

2.2 Prostate cancer biomarkers

Biochemical biomarkers are indicators of biological and pathological processes that can be measured with relative objectivity. Prostate cancer biomarkers are most commonly detected from patients' blood, urine or tissue biopsy samples and can be utilized in diagnosis, estimating prognosis, predicting response to treatments, and detecting recurrence after curative treatment (Jakobsen et al. 2016). Due to heterogenous nature of prostate cancer, current biomarkers suffer from low diagnostic specificity and are not good enough in distinguishing indolent tumors from aggressive ones in the early stages of the disease (Jakobsen et al. 2016). Combinations of different urine or blood based biomarkers, such as TMPRSS2:ERG, PCA3 and PSA, and quantification of multiple genes or proteins from biopsy tissue samples are emerging as more powerful predictors (Mohler et al. 2016; Tomlins et al. 2016). Safety, easiness of use, low cost, reproducibility and convenience to patient are also important considerations that effect adoption of biomarker based tests in to clinical use.

2.2.1 Biomarkers clinically available for prostate cancer diagnosis and prognosis

PSA: PSA, or human kallikrein 3, is a protein secreted by prostate epithelial cells into seminal fluid (Lilja H 1985). PSA is detectable in blood samples and its levels are elevated in prostate cancer as well as other conditions such as benign prostatic hyperplasia (Savblom et al. 2005). Although utilized earlier in monitoring treatment response and cancer recurrence, it was adopted in widespread diagnostic use in early 1990s and was approved by Food and Drug Administration (FDA) in 1994, which resulted in considerable increase in detection of early-stage disease through increased number of prostate biopsies (Potosky et al. 2001). As stated earlier, part of the decrease in prostate cancer mortality can be attributed to widespread PSA testing (Kvale et al. 2007; Schroder et al. 2014). Its non-specificity, and in particular, the inability to distinguish docile cancer cases from aggressive ones, has led to over-treatment causing personal harm and increase in health-care expenses, which is in part why new biomarkers are actively sought (Schroder et al. 2014). PSA is well established and still the first line biomarker for prostate cancer detection. Due to its association with tumor volume and Gleason grade, and recent findings linking early age PSA levels to later development of metastatic disease, PSA will most likely continue to be utilized along with other markers in risk-stratification (Pinsky et al. 2007; Vickers et al. 2013).

Several improvements upon standard PSA testing have been proposed with some success. PSA velocity, which stands for increase rate of patient's PSA levels between measurements, has been shown to be more accurate than single PSA measurement in predicting aggressive cancer cases, but the studies are controversial (Orsted et al. 2013; Vickers et al. 2011). Prostate health index (PHI) is calculated by formula: ([-2]proPSA/free PSA) × √PSA, taking into account inactive precursor PSA ([-2]proPSA), activated free PSA and total PSA. There is considerable evidence that PHI improves predictive value upon standard PSA testing and could reduce unnecessary biopsies (Catalona et al. 2011; Jansen et al. 2010). PHI has been FDA approved since 2012. PSA density, where PSA levels are compared to the prostate volume, is reasoned to better distinguish between benign prostate hyperplasia and prostate cancer. Although earlier studies on the performance of PSA density are somewhat controversial and the sizing of prostate poses a challenge, a recent study has shown a significant increase in specificity when compared to use of free and total PSA (Cookson et al. 1995; Verma et al. 2014). Another prostate-specific protein, human kallikrein 2 (hK2) is a serine protease that converts inactive pro-PSA to active PSA (Kumar et al. 1997). Its increased expression is more

specific to malignant conditions of the prostate and to high-grade disease than that of PSA's (Darson et al. 1997). A panel combining total PSA, free PSA, intact PSA and hK2 has shown promising results in specificity to high-grade cancers and could possibly reduce unnecessary biopsies and over-treatment of low-risk tumors (Benchikh et al. 2010; Vickers et al. 2008).

PCA3: PCA3 is a non-coding mRNA specific to prostate. It is overexpressed in prostate cancer cells and can be detected from urine samples after DRE (Hessels and Schalken 2009). An FDA approved PCA3 assay is available for use in deciding on repeat biopsies after previous negative biopsies (Groskopf et al. 2006). PCA3 has limited value in detecting aggressive cancer cases, but it has shown to be an accurate predictor of indolent prostate cancer, and its use in selecting patients for active surveillance could be beneficial (Auprich et al. 2011).

TMPRSS2-ERG fusion: As stated previously, TMPRSS2-ERG fusion is present in approximately half of prostate cancer cases and is associated with cancer-specific death and metastatic spread, although contradictory results have also been published (Tomlins et al. 2009). TMPRSS2-ERG mRNA transcript can be detected from urine after DRE, and its use as a biomarker has been shown to increase specificity and prognostic capability of PSA based methods and PCA3 measurements (Leyten et al. 2014)

ConfirmMDx: ConfirmMDx is a test based on the data from a next-generation sequencing study reporting prognostic value of epigenetic alterations in prostate cancer (Gu et al. 2015). It analyses methylation status of three genes: *GSTP1*, *RASSF1*, and *APC*, from negative biopsy tissue samples, and has been shown to have an 88% negative prediction value on repeat prostate biopsies in clinical setting (Partin et al. 2014). It has also been shown to improve the detection of high-grade cancer cases from negative biopsies, when combined with traditional risk stratification involving PSA levels and DRE (Van Neste et al. 2016).

Gene panels: Oncotype DX is a gene test that measures expression levels of 12 prostate cancer-related and 5 housekeeping genes from needle biopsy samples with quantitative RT-PCR. Genes were selected from multiple prostate cancer-related pathways, based on results from prostatectomy and biopsy studies, with focus on genes associated with clinical recurrence, prostate cancer death, and adverse pathology at prostatectomy (Klein et al. 2014). Two independent studies have shown that the gene panel is a strong predictor of high-grade cancer and recurrence after radical prostatectomy (Cullen et al. 2015; Klein et al. 2014).

Prolaris is another gene panel used for prostate cancer risk stratification that utilizes quantitative RT-PCR measurements of 31 cell cycle-related genes and 15 housekeeping controls from needle biopsy samples. The test has been shown to independently predict prostate

cancer-specific mortality, biochemical recurrence after treatment, and metastasis (Cooperberg et al. 2013; Cuzick et al. 2015).

Circulating tumor cells: Shedding of cells into the circulation is considered to be a property of the tumor that is associated with characteristics, other than the extent of cancer, conferring poor prognosis (Danila et al. 2011). Circulating tumor cells (CTC) can be counted with FDA approved Veridex CellSearch assay and detected high cell count is a reliable prognostic factor for poor survival time (Scher et al. 2009). The decrease in CTC count in response to first line chemotherapy of mCRPC is also strongly associated with longer survival time, and can be used to monitor treatment efficacy with greater predictive value than PSA decrease (Danila et al. 2011; Scher et al. 2009). Use of CTC in clinical practice is hindered by poor detection rate of current methods, which is probably the cause of substantial variation in survival times of patients with small CTC count (Danila et al. 2014).

In addition to enumeration, isolated CTCs can be molecularly profiled to gain insight of characteristics of the tumor of origin (Holcomb et al. 2009). CTC heterogeneity makes this approach less straightforward and it is still in experimental stages. A study showing that AR splice variant 7 mRNA (AR-V7) could be detected in CTCs, found that CRPC patients positive with the marker had worse treatment response to abiraterone and enzalutamide and had worse progression-free survival (Antonarakis et al. 2014). AR-V7 lacks the ligand-binding domain targeted by abiraterone and enzalutamide, but remains constitutively active. A study subjecting CTCs acquired from patients with metastatic prostate cancer to pathologic review, found that detection of CTCs with very small nucleus was associated with visceral metastases (Chen et al. 2015). It was also noted that CTCs with very small nucleus were identified before the detection of visceral metastatic lesions, suggesting prognostic role for this type of approach.

2.2.2 Promising biomarker research

MicroRNAs: As stated earlier, widespread deregulation of microRNAs in prostate cancer has been reported compared to normal prostate tissues and altered microRNA expression profiles have been associated with prostate cancer properties, such as aggressiveness, metastasis, and recurrence (Martens-Uzunova et al. 2012; Tong et al. 2009). They are ideal as biomarkers as they can be measured from urine, blood, or tissue samples and they are stable molecules relatively easy to detect and quantify (Chen et al. 2008).

Expression profiles of miR-221 have been shown to correlate with metastasis formation, recurrence risk after radical prostatectomy, tumor progression, and Gleason score (Kneitz et al. 2014; Spahn et al. 2010; Yaman Agaoglu et al. 2011). Prostate cancer cell line

studies indicate miR-221 as a tumor suppressor regulating cell growth, apoptosis, and invasiveness (Kneitz et al. 2014). A combination of expression of four microRNAs: miR-96-5p, miR-183-5p, miR-145-5p, and miR221-5p, measured from tissue samples has been shown to outperform PSA in predicting diagnosis and to significantly predict tumor aggressiveness, metastasis, and overall survival of patients (Larne et al. 2013). The miR-21 is a known onco-miR deregulated in several types of cancer and upregulation of miR-21 plasma levels is associated to development of castration-resistant prostate cancer (Selcuklu et al. 2009; Shen et al. 2012). It is regulated by AR and it has been shown to promote both hormone-dependent and hormone-independent prostate cancer growth (Ribas et al. 2009). Expression levels of miR-21 have also been shown to predict treatment response to docetaxel-based chemotherapy (Zhang et al. 2011). Diagnostic and prognostic use of microRNAs in prostate cancer is founded in increasing experimental evidence, and consensus is forming on the microRNAs with most potential. As with all emerging biomarkers, differences in study design, sample collection, and in the techniques used in analysis should be minimized with development of proper guidelines (Fabris et al. 2016).

Nucleic acids in peripheral blood: Measuring of free circulating nucleic acid levels from peripheral blood, or mRNA levels in whole blood samples, can be used to characterize prostate cancer tumors. Interestingly, increase in total cell-free DNA in peripheral blood has been shown to associate with PSA-recurrence after radical prostatectomy (Bastian et al. 2007). A six-gene panel, measured with quantitative RT-PCR from peripheral blood of patients with CRPC, was shown to be able to group patients to low-risk group with a median survival of more than 34.9 months, and to high-risk group with median survival of 7.8 months (Ross et al. 2012). Another study utilized microarray analysis of over 47 000 transcripts from whole blood to categorize patients in to four prognostic groups based on expression profiles, with one of the groups significantly associated with CRPC and poor survival (Olmos et al. 2012). A nine-gene panel was then pinpointed, which could reliably categorize patients into these groups.

In an attempt to complement CTC count in prognosis of CRPC, a five-gene panel was created based on high expression in CRPC cases and prostate-specificity (Danila et al. 2014). Detection of two or more of these genes from whole blood samples could predict overall survival with similar accuracy to CTC count, and cases with poor survival despite a favorable CTC count were also identified. Another study searching surrogates for CTC count utilized peripheral blood mononuclear cell (PBMC) fraction. *KLK3*, *PCA3*, and *TMPRSS2-ERG* mRNAs were detected in 89%, 53%, and 37% of CRPC patients' PBMC fraction, respectively, while healthy controls were negative for all markers (Dijkstra et al. 2014). Expression levels of

all the markers were found to be reduced in response to docetaxel treatment, suggesting this approach as candidate for treatment monitoring. A recent study has shown that AR-V7 can be directly quantified from peripheral whole blood samples to predict the progression-free survival of CRPC patients receiving abiraterone or enzalutamide treatment (Seitz et al. 2017).

Copy-number aberrations: As stated earlier, CNAs are important and common mechanisms in prostate carcinogenesis, and overall CNA burden and specific CNAs could have prognostic potential. A study analyzing CNA burden as the percentage of the tumor genome affected by CNA found that it was significantly associated with biochemical recurrence and metastasis after radical prostatectomy (Hieronymus et al. 2014). The study also states that CNA burden can be measured in needle biopsy samples using low-input whole-genome sequencing, which would make it a more accessible prognostic option. Another study utilizing whole genome analysis pinpointed deletion of 8p23 as associated with advanced stage, and gain at 11q13.1 predicting recurrence independently of cancer stage and grade (Paris et al.2004). In a more elaborate approach, unsupervised hierarchical clustering of tumors based on CNA data produced tumor subgroups with differing risk of relapse after radical prostatectomy. The difference in risk could not be explained by differential expression of individual genes (Taylor et al. 2010). These studies display the prognostic potential of CNA analysis, but additional validation and standardization of methods are needed before it can be utilized in clinical settings.

Exosomal biomarkers: Prostasomes are microvesicles produced by prostate acinar cells, normally released into seminal fluid. They have been found to be present in peripheral circulation of men with prostate cancer and their amount has been shown to correlate with Gleason score (Tavoosidana et al. 2011). Exosomal vesicles are stable and their RNA content is protected from exogenous RNAses. Vesicles' molecular composition is considered to reflect composition of their cellular origin (Zijlstra and Stoorvogel 2016). These properties make them attractive for extraction of prostate cancer-specific markers and for example *TMPRSS2:ERG* and *PCA3* RNAs have been detected from exosomal fractions from the urine of prostate cancer patients (Nilsson et al. 2009). MicroRNAs with biomarker potential can also be harvested from microvesicles and exosomes. This was utilized in study that compared microRNA content in serum-derived vesicles between recurrent and non-recurrent cancer cases following radical prostatectomy and found miR-141 and miR-375 to be associated with metastatic prostate cancer (Bryant et al. 2012).

2.2.3 Short and long-term future of biomarkers

The most established new urine and blood based biomarkers are close to have accumulated enough data to replace or to complement PSA testing as a standard method to decide whether to proceed to prostate biopsy. Thorough comparison of the different approaches is still needed to ensure that the new guidelines best utilize the capabilities of new biomarkers to reduce prostate cancer mortality and adverse effects of overtreatment. Tissue based tools, such as gene panels, could possibly be utilized more extensively to verify negative biopsies, and to assign patients with higher risk for recurrence after curative treatment to closer follow-up.

Promising research on gene panels and microRNAs suggest that they might prove important predictors and monitors for treatment efficacy. This could help to decide the most effective treatment modalities for both localized cancer and for mCRPC. Better treatment targeting will reduce side effects caused to patients and limit health care expenses. Possibly the highest hopes are set on microRNAs for their versatility, and because their expression levels reflect multiple prostate cancer-related pathways.

3 AIMS OF THE THESIS

The goal of this Master's thesis study was to assess the possible role of *OXGR1* in prostate cancer. RNA sequencing data of clinical prostate cancer samples show significant overexpression of *OXGR1* in small subset of samples. There are yet no studies proposing *OXGR1* as a potential oncogene, but preliminary data of this group suggests that *OXGR1* overexpression could confer growth advantage to prostate cancer cells. The aims of this study were:

1) To verify the overexpression in prostate cancer cells with stable and transient *OXGR1* transfections at RNA and protein level.
2) To corroborate the results of preliminary experiments showing possible growth advantage in *OXGR1* overexpressing cells.
3) To investigate possible mechanisms by which *OXGR1* expression and protein coded by it, OXGR1, could confer growth advantage. This is performed by introducing *OXGR1* expressing cells to growth media containing different concentrations of key substrates.

4 MATERIALS AND METHODS

4.1 Cell culture methods

The study was carried out using two prostate cancer cell lines: PC-3 and LNCaP, which were obtained from American Type Culture Collection (ATCC). Stable *OXGR1* expressing PC-3 and LNCaP cell lines were created previously by transfecting cells with pcDNA3.1 vectors (Invitrogen) containing *OXGR1* and neomycin resistance gene under constituve promoter. Three *OXGR1* transfected PC-3 cultures (PC-3 OXGR1_4, PC-3 OXGR1_8 and PC-3 OXGR1_10) were chosen for the study based on previous results along with two cultures transfected with empty vectors (PC-3 pcDNA_1 and PC-3 pcDNA_3). Likewise, three *OXGR1* transfected LNCaP cultures (LNCaP OXGR1_2, LNCaP OXGR1_6 and LNCaP OXGR1_13 were chosen for the study along with two cultures transfected with empty vectors (LNCaP pcdna_2 and LNCaP pcdna_4).

PC-3 and LNCaP cells were cultured according to ATCC guidelines in Ham's F12 medium with 10% fetal bovine serum and in RPMI 1640 medium with 10% fetal bovine serum (Gibco, Life Technologies) respectively. Vector transfected lines were kept under constant selection pressure with 0.2 mg/ml Geneticin® (Thermo Fisher Scientific).

4.2 Transient transfection

A transient transfection was performed to PC-3 cells with *OXGR1* cDNA in pEZ-MO2 vector (GeneCopoeia). pcDNA3.1 vectors were transfected as controls. Transfection was carried out using jetPei (Polyplus Transfection) according to manufacturer's instructions with complex formation incubation time at 15 minutes.

4.3 RT-qPCR

Total RNA was extracted from pelleted cells harvested at ~80% confluence using TRI Reagent® (Sigma) following manufacturer's guidelines. RNA concentrations were measured using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription of RNA to cDNA was performed with Maxima reverse transcriptase with random hexamer primers (Thermo Fisher Scientific).

OXGR1 RNA levels were quantified using standard curve derived from total RNA pooled from prostate cancer cell lines PC-3, 22Rv1 and LNCaP. *OXGR1* levels were further normalized to housekeeping gene TATA-binding protein (TBP). Primer sequences for *OXGR1* were GCTGAACCTGGCCTGCACAGA forward and TGATCCACACCACAGCACAGGC

reverse, and for TBP GGGGAGCTGTGATGTGAAGT forward and GAGCCATTACGTCGTCTTCC reverse. Quantitative PCRs were performed with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) following manufacturer's guidelines with optimized annealing temperature for both primer pairs. Equipment used was CFX96 q-RT-PCR detection system (Bio-Rad). Proper amplification was ensured by agarose gel electrophoresis of PCR products. Data analysis was performed with Excel software.

4.4 Cell lysates, SDS-PAGE, and western blot

Protein content was extracted from ~80% confluent cells using triton lysis buffer (0.5% Triton-X 100, 300 mM NaCl in 50 mM Tris-HCL pH 7.4) supplemented to contain 1x Complete protease inhibitor cocktail (Roche), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and sonication with Bioruptor (Diagenode). Protein concentration was measured using DC Protein Assay (BioRad) and EnVision® 2104 multilabel reader. Bovine serum albumin (BSA) dilution series was used as standard.

30 - 50 µg of protein lysate (same amount of protein in each sample of a single gel) was combined with Red Loading Buffer and DTT (New England Biolabs), and loaded to 10% SDS-PAGE gel. Kaleidoscope™ Prestained SDS-PAGE Standard (Bio-Rad) was also loaded into each gel. Gel was run on Bio-Rad equipment first ~30 min. at 50 V, then ~120 min. at 120V. Proteins in the gel were then transferred to Immobilon P membranes using The Mini Trans-Blot® cell (Bio-Rad) according to manufacturer guidelines. The proper transfer time was optimized to ~45 min. at 50 V.

Unspecific binding was blocked in 3% BSA in phosphate-buffered saline (PBS) for 1h RT, and washes were performed in PBS with 0.1% Tween-20. Membranes were probed with primary monoclonal anti-OXGR1 [EPR6305(2)] rabbit antibody (Abcam) for 1 h RT at 1:500 dilution and with secondary polyclonal horseradish peroxidase (HRP)-conjugated swine anti-rabbit antibody (Dako) for 30 min RT at 1:5000 dilution. Monoclonal pan-actin Ab-5 mouse antibody (Thermo Fisher Scientific) was used at 1:1000 dilution along with polyclonal HRP-conjugated rabbit anti-mouse antibody (Dako) at 1:5000 dilution to verify even total protein content. Western Blotting Luminol Reagent SC-2018 (Santa Cruz) was then applied to membranes. X-ray films were exposed to membranes for 5 seconds to 10 minutes depending on the antibody and were developed using CP1000 table top film processor (Agfa).

4.5 Immunofluorescence staining

For immunofluorescence staining, cells were seeded on coverslips to achieve ~80% confluence over 3 days. The day following the seeding, transient *OXGR1* transfection was performed on part of the samples (see before). Third day from seeding, coverslips were rinsed with PBS and fixed with 4% paraformaldehyde 30 min RT. For antibody probing, cells were permeabilized with 0.5% NP-40 in PBS for 5 min RT. Unspecific binding was blocked in 3% BSA in PBS for 10 min RT. Antibodies used were primary monoclonal anti-OXGR1 [EPR6305(2)] rabbit antibody (Abcam) for 1 h RT at 1:250 dilution and secondary fluorescent labeled Alexa Fluor 594 goat-anti-rabbit antibody (Invitrogen) for 1 h RT at 1:100 dilution. Coverslips were mounted on microscope slides with Vectashield mounting media (Vectro Labs) containing DAPI. Cells were imaged with Zeiss Axio Imager M2 fluorescence microscope.

4.6 Cell proliferation experiment

For proliferation assay, the cells were seeded on 24-well plates as four replicates. Number of cells seeded was 15 000 cells / well for PC-3 cells and 30 000 cells / well for LNCaP cells. This was assigned day 0. At day 1 cells regular medium was adjusted to experimental conditions, and transient *OXGR1* transfection was performed on part of the samples (see before) (Table 2).

Table 2: Different media used for cells in proliferation assays. Regular medium defined above for different cell types.

Experiment	Medium
Varying alpha-ketoglutarate	Regular medium with added alpha-ketoglutarate to final concentrations of 0 µM, 25 µM, 75 µM and 1000 µM.
Varying glucose / glutamine	DMEM, low glucose (Gibco) adjusted to different conditions **Normal**: 2.5 g/l glucose and 2 mM L-glutamine **Low glucose**: 1 g/l glucose and 2 mM L-glutamine **High glucose**: 4.5 g/l glucose and 2 mM L-glutamine **Low glutamine**: 2.5 g/l glucose and 0 mM L-glutamine **High glutamine**: 2.5 g/l glucose and 4 mM L-glutamine
Transient *OXGR1* transfection	Regular medium

Cells were imaged at indicated days with Olympus IX71 microscope, OASIS automation control system and Surveyor imaging software (Objective imaging Ltd.). Cell surface area was measured with an in-house macro for ImageJ software.

4.7 Small interfering RNA knockdown of *OXGR-1*

OXGR-1 expression knockdown in stably expressing LNCaP cells was performed with two targeting sequences with Silencer Select Pre-designed siRNA (Ambion). 150 000 cells were seeded on 6 well plates and transfection mixture of 500 µl Optimem (Gibco), 0.9 µl of 20 µM stock of two siRNA molecules and 9 µl of INTERFERin® (Polyplus Transfection) was added to cells after 10 minutes of complex formation at RT. A non-targeting siRNA was used as a control. Cells were harvested at 72 h for protein and RNA extraction.

5 RESULTS

5.1 *OXGR1* expression quantification by RT-qPCR

pcDNA3.1 vectors (Invitrogen) were used to constitutively overexpress *OXGR1* in prostate cancer cell lines PC-3 and LNCaP. Overexpression at mRNA level was confirmed by using RT-qPCR. Expression levels of *OXGR1* in different transfection lines and controls are shown in Figure 4. Expression in cell lines transfected with empty vector and in non-transfected

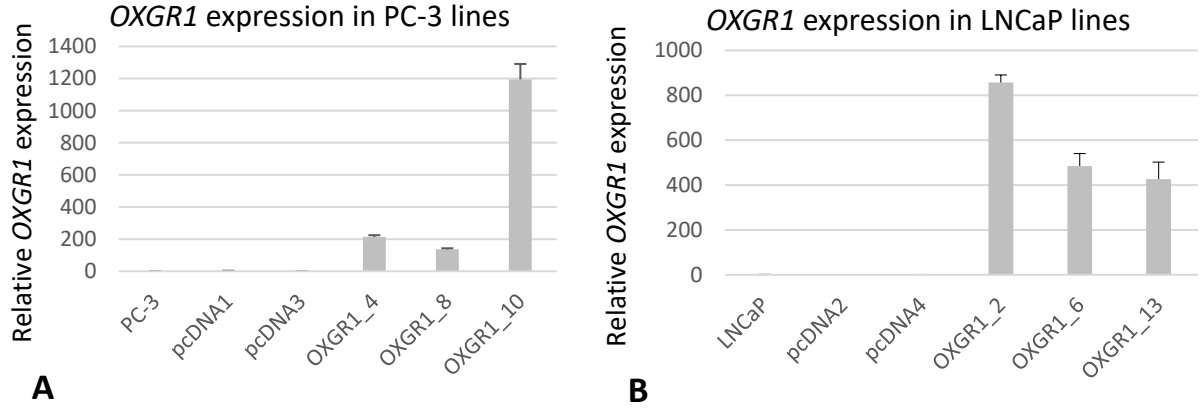

Figure 4: A) Expression of *OXGR1* mRNA in PC-3 cells with stable *OXGR1* transfection, with empty expression vector, and non-transfected control. B) Expression of *OXGR1* mRNA in LNCaP cells with stable *OXGR1* transfection, with empty expression vector, and non-transfected control. Error bars represent standard deviations of RT-qPCR replicates.

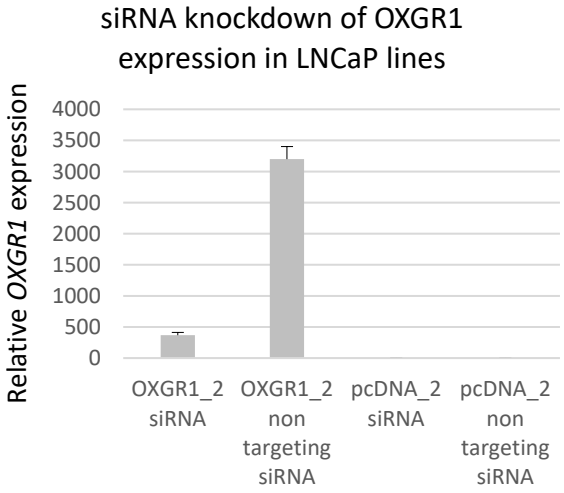

Figure 5: Expression of *OXGR1* mRNA in LNCaP cells with stable *OXGR1* overexpression knocked down with siRNA and non-targeting control. LNCaP pcDNA_2 as non-transfected control. Error bars represent standard deviations of RT-qPCR replicates.

Table 3: Relative *OXGR1* expression levels of non-transfected cell lines

Cell line	Relative *OXGR1* expression	Standard deviation
PC-3	0.78	0.40
PC-3 pcDNA_1	2.20	1.27
PC-3 pcDNA_3	0.38	0.16
LNCaP	1.13	0.89
LNCaP pcDNA_2	0.52	0.11
LNCaP pcDNA_4	0.86	0.66

controls is virtually non-existent, while cell lines with stable *OXGR1* transfection show hundred- to thousand-fold increase in expression at mRNA level. PC-3 OXGR1_10 shows over five folds higher expression than other transfected PC-3 clones, and LNCaP OXGR1_2 shows almost two folds higher expression than other transfected LNCaP clones. Minor expression was also measured

in non-transfected cell lines, both PC-3 and LNCaP (table 3). siRNA targeting *OXGR1* mRNA was used to knockdown *OXGR1* expression at mRNA level. The knockdown effect was measured with RT-qPCR (Figure 5). siRNA transfection decreased *OXGR1* expression significantly: approximately 9 folds reduction in LNCaP OXGR1_2 cell line was detected. Despite this, the expression stayed well above the expression of controls transfected with empty vector. Thus, a partial knockdown was achieved.

5.2 Verification of OXGR1 overexpression by western blotting

Western blot experiments were performed to cells to confirm OXGR1 expression at protein level and to semi-quantitate protein concentration in different cell lines. Protein content of cells were probed with anti-OXGR1 and monoclonal anti-pan-actin antibodies to verify OXGR1 expression at protein level and to semi quantitatively compare expression levels between samples. PC-3 cells with stable *OXGR1* transfection show a clear but weak band when probed with OXGR1 antibody (Figure 6). Based on the molecular marker the band contains protein with molecular weight above 50 kilodaltons, while the predicted band size for OXGR1 is approximately 38 kilodaltons. Pan-actin probing shows relatively even protein content between samples. There is no difference in OXGR1 antibody band intensity between cells transfected with *OXGR1* and cells transfected with empty vector. The results are similar when regular PC-

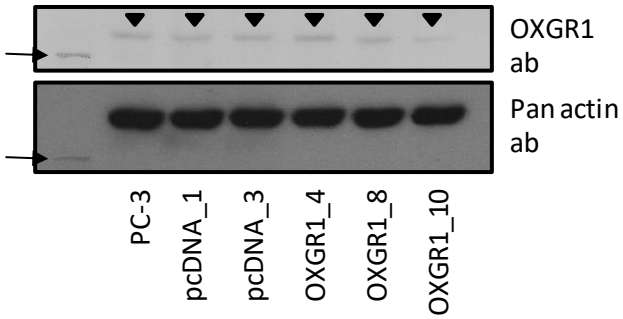

Figure 6: Western blot showing protein from regular and transfected PC-3 cells, probed with OXGR1 and pan-actin antibodies. Arrows mark 45.7 kilodalton band on molecular marker. Arrowheads mark OXGR1 bands.

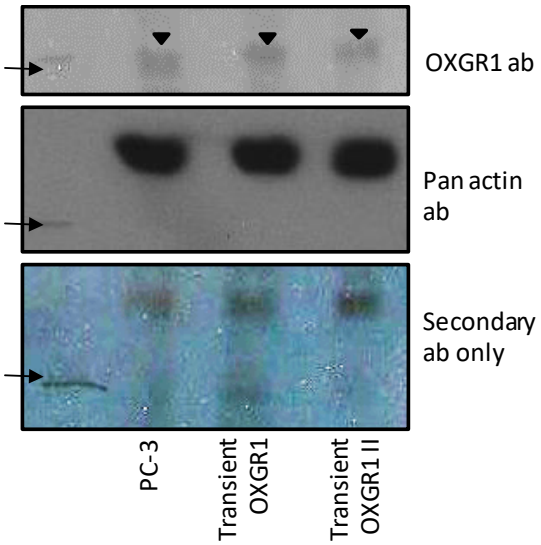

Figure 7*:* Western blot showing protein from regular and transiently transfected PC-3 cells, probed with OXGR1 and pan-actin antibodies. Arrows mark 45.7 kilodalton band on molecular marker. Arrowheads mark OXGR1 bands.

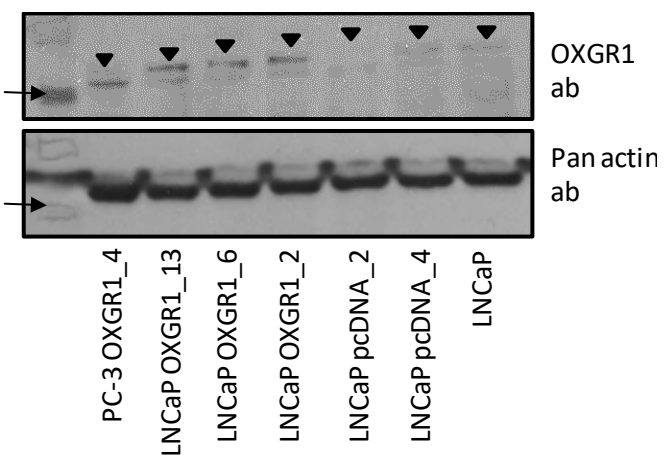

Figure 8*:* Western blot showing protein from regular and transfected LNCaP cells along with one transfected PC-3 cell line, probed with OXGR1 and pan-actin antibodies. Arrows mark 45.7 kilodalton band on molecular marker. Arrowheads mark OXGR1 bands or places where the band would be.

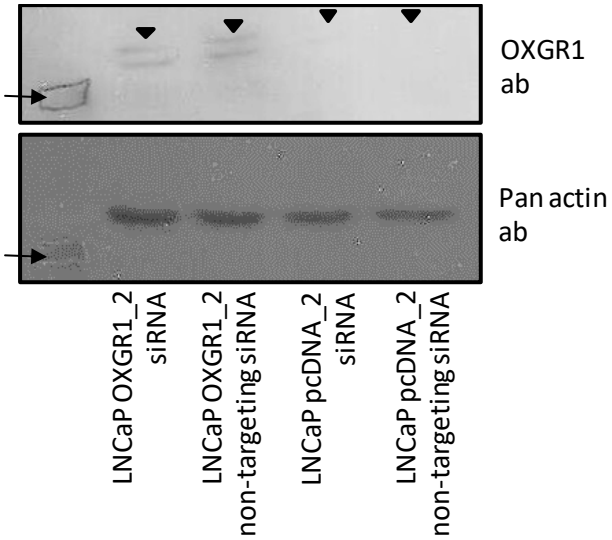

Figure 9*:* Western blot showing protein from transfected LNCaP cells with siRNA treatment, probed with OXGR1 and pan-actin antibodies. Arrows mark 45.7 kilodalton band on molecular marker. Arrowheads mark OXGR1 bands or places where the band would be.

3 cells are compared to cells with transient OXGR1 transfection (Figure 7). Here is also shown a probing with only the secondary antibody to verify that OXGR1 band is result from primary antibody binding.

LNCaP cells with stable *OXGR1* transfection show higher intensity bands when probed with OXGR1 antibody than regular LNCaP cells and cells transfected with empty vector (Figure 8). Here, the band seems to have even a higher molecular weight than that seen with PC-3 cells, and two bands with minor difference in molecular weight can be seen. LNCaP cells with stable *OXGR1* transfection treated with anti-*OXGR1* siRNA have slightly weaker staining than those treated with non-targeting siRNA, while the pan-actin probing shows even signal (Figure 9). With all LNCaP OXGR1 antibody probings, formation of double bands can be detected.

5.3 OXGR1 protein expression and localization analysis by immunocytochemistry

To further study the expression and cellular localization of OXGR1, PC-3 cells were analyzed by immunocytochemistry. Approximately 30% of the transiently transfected PC-3 cells show increased OXGR1 expression, but similar effect can also be seen on the empty vector control sample (Figure 10). In PC-3 cells with stable *OXGR1* transfections, the cell to cell differences in OXGR1 expression seem to be lower than those with transient transfections (Figure 11),

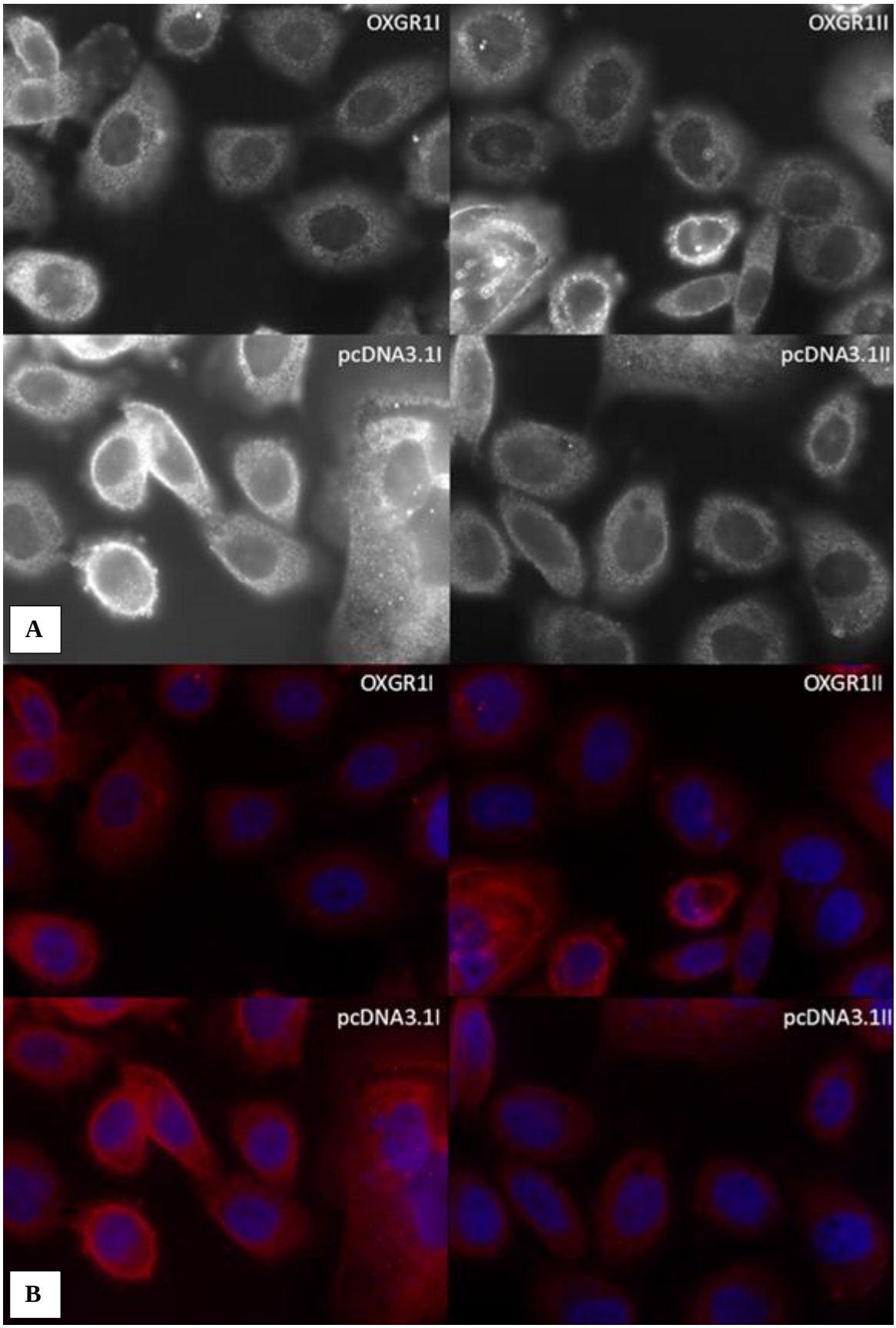

Figure 10: A) Immunofluorescent anti-OXGR1 staining of transiently transfected PC-3 cells (OXGR1I and OXGR1II) and cells transfected with empty vector (pcDNA3.1I and pcDNA3.1II). B) Same images merged with nuclear DAPI staining.

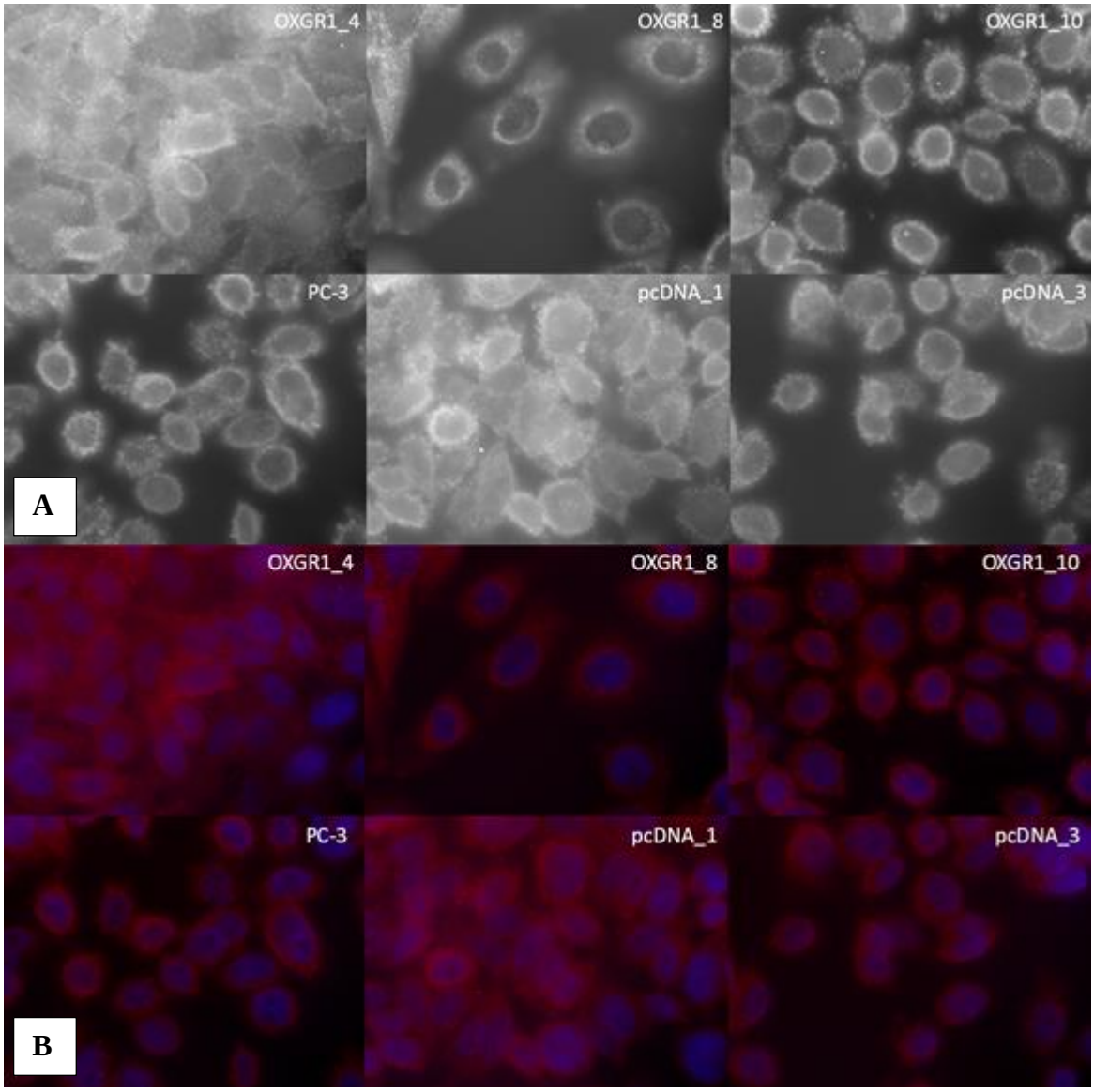

Figure 11: A) Immunofluorescent anti-OXGR1 staining of PC-3 cells with stable *OXGR1* transfection (OXGR1_4, OXGR1_8 and OXGR1_10), cells transfected with empty vector (pcDNA_1 and pcDNA_3) and non-transfected PC-3 cells (PC-3) B) Same images merged with nuclear DAPI staining.

although cells with higher expression levels can be seen. Cells with higher expression levels can also be seen in the control series. OXGR1 is seen to localize mainly to the cell membrane and to lesser extent to the cytoplasm. All images are acquired from representative cell areas.

5.4 Cell proliferation experiment

Cell proliferation experiments were carried out to study whether *OXGR1* overexpression provides growth advantage to PC-3 and LNCaP cells. With transiently transfected PC-3 cells, a minor increase in growth area is seen compared to the cells transfected with empty vector (Figure 12). To study whether metabolic conditions affect the growth of *OXGR1* expressing cells, cell proliferation experiments were carried out in media containing different

concentrations of key metabolites along with regular growth medium. Stable *OXGR1* transfection in PC-3 cells didn't increase growth rate in comparison to cells transfected with empty vector in growth experiments with varying glucose and glutamine concentrations (Figure 13). One exception to this is the experiment with low glutamine concentration, where cell line OXGR_8 has increased growth compared to all other cell lines (Figure 13). In an experiment with varying alpha-ketoglutarate (AKG) concentrations, no clear differences can be seen in growth rate between *OXGR1* transfected and control PC-3 cell lines (Figure 14). In the same AKG experiment with LNCaP cell lines, minor increase in growth rate of lines with stable *OXGR1* transfection is seen, when AKG is present in the medium (Figure 15).

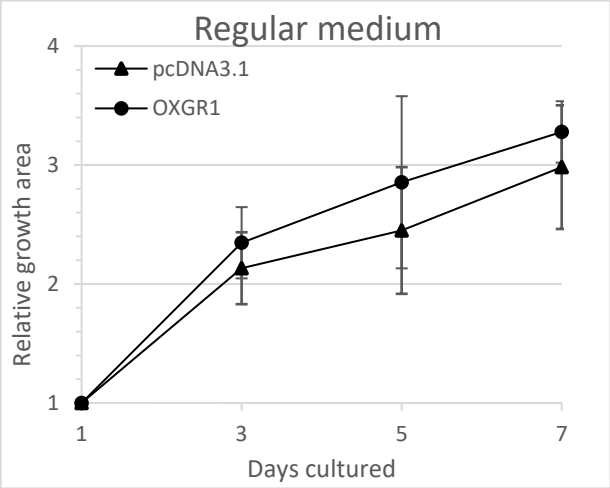

Figure 12: Relative growth of transiently transfected PC-3 cells (*OXGR1*) and cells transfected with empty vector (pcDNA3.1). Error bars represent standard deviations between replicates.

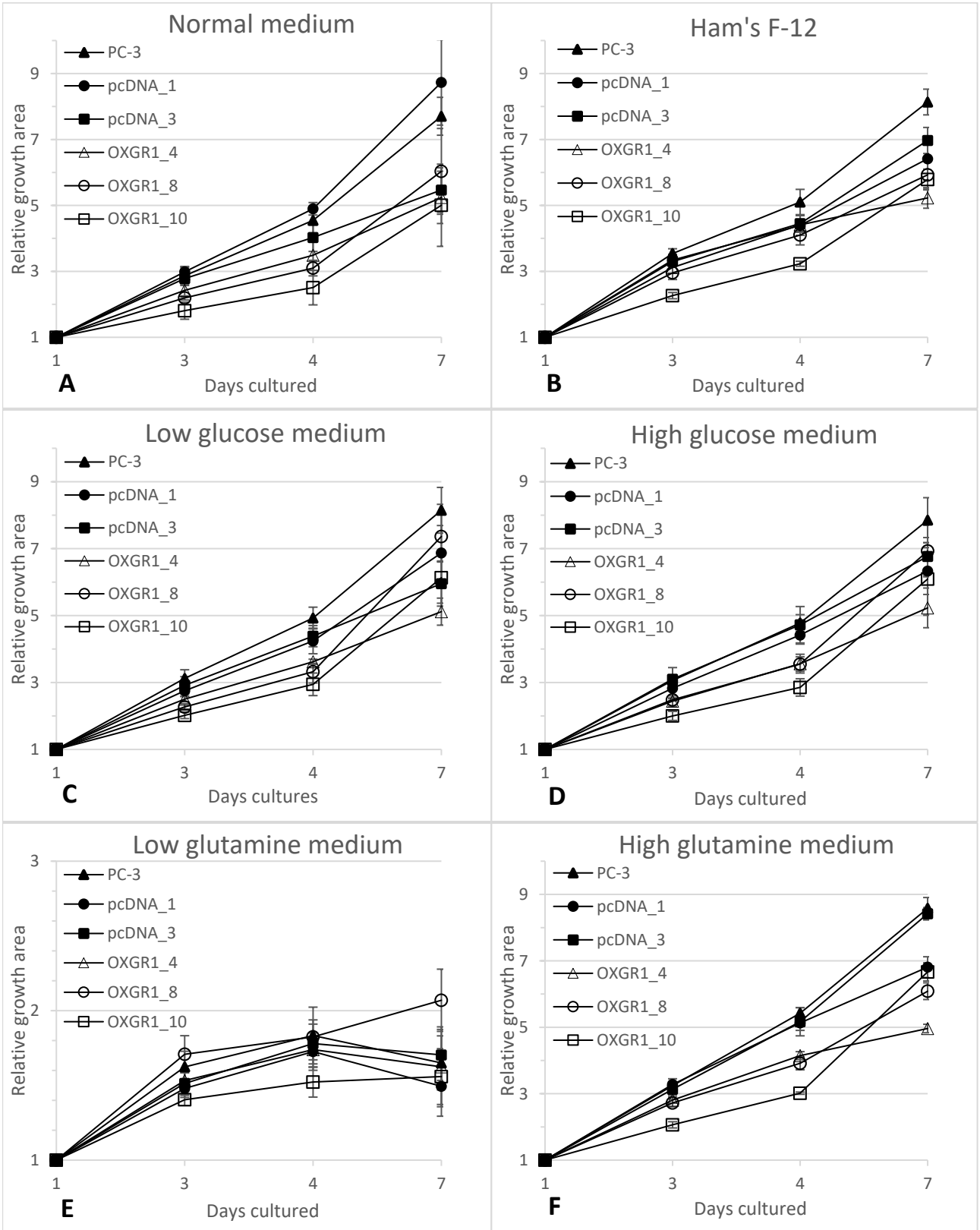

Figure 13: Relative growth of PC-3 cells with stable *OXGR1* transfection (OXGR1_4, OXGR1_8 and OXGR1_10) in regular growth medium and media containing different concentrations of glucose and glutamine. A) Normal medium B) Ham's F-12 C) Low glucose medium D) High glucose medium E) Low glutamine medium F) High glutamine medium. Media compositions are listed in table 1. Non-transfected PC-3 cells (PC3) and cells transfected with empty vector (pcDNA1 and pcDNA3) serve as controls. Error bars represent standard deviations between replicates.

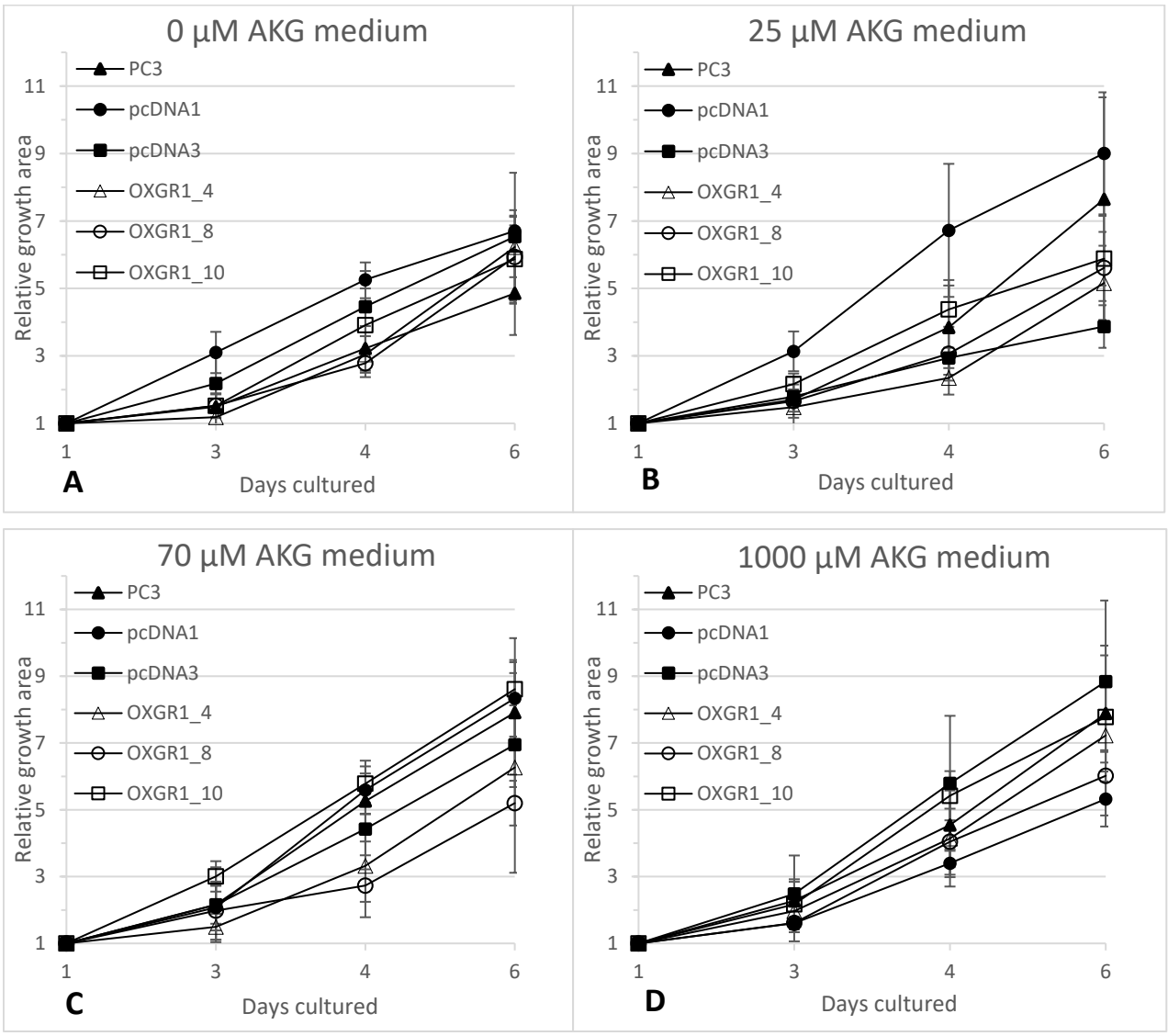

Figure 14: Relative growth of PC-3 cells with stable *OXGR1* transfection (OXGR1_4, OXGR1_8 and OXGR1_10) in growth media containing different concentrations of AKG. A) 0 µM AKG medium B) 25 µM AKG medium C) 70 µM AKG medium D) 1000 µM AKG medium. Non-transfected PC-3 cells (PC3) and cells transfected with empty vector (pcDNA1 and pcDNA3) serve as controls. Error bars represent standard deviations between replicates. AKG = alpha-ketoglutarate.

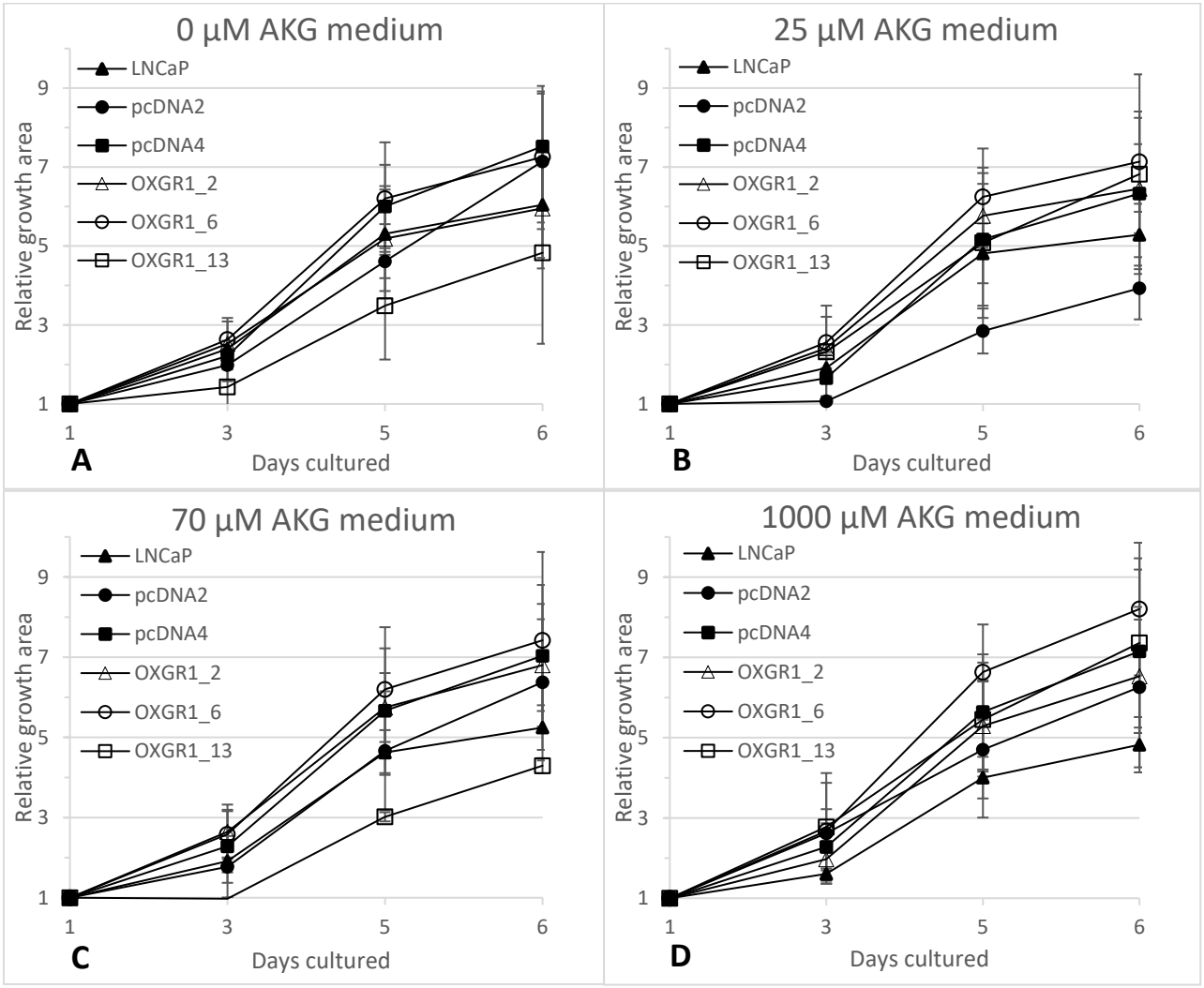

Figure 15: Relative growth of LNCaP cells with stable *OXGR1* transfection (OXGR1_2, OXGR1_6 and OXGR1_13) in growth media containing different concentrations of AKG. A) 0 µM AKG medium B) 25 µM AKG medium C) 70 µM AKG medium D) 1000 µM AKG medium. Non-transfected LNCaP cells (LNCaP) and cells transfected with empty vector (pcDNA2 and pcDNA4) serve as controls. Error bars represent standard deviations between replicates. AKG = alpha-ketoglutarate.

6 DISCUSSION

OXGR1 codes for a G-protein coupled receptor OXGR1, which is expressed mainly in human kidney, placenta, lung, trachea, salivary glands, eosinophils, mast cells, and nasal mucosa (Kanaoka et al. 2013; Shirasaki et al. 2017; Wittenberger et al. 2002). The receptor is activated by its ligands AKG and cysteinyl leukotrienes, particularly Leukotriene E_4 (LTE_4) (He et al. 2004; Kanaoka et al. 2013). Studies on the biological function of OXGR1 are yet limited, but some propositions have been made. It has been shown that AKG levels respond to acid-base stress in mouse kidney tubules and, in turn, cause secretion of bicarbonate through activation of OXGR1 to further buffer the urea (Tokonami et al. 2013). Effects mediated through LTE_4 seem to play a role in hypersensitivity immune reactions, causing vascular leakage particularly in epithelial smooth muscle cells (Kanaoka et al. 2013; Shirasaki et al. 2017).

OXGR1 was chosen as the target gene of this study on the grounds of unpublished data of this group showing high overexpression of *OXGR1* at RNA level in a small subset of clinical prostate cancer samples. There is no published data suggesting *OXGR1* to be a cancer promoting factor. Preliminary cell proliferation experiments also showed increased growth rate of PC-3 cells with both stable and transient *OXGR1* transfection. The purpose of this study was to verify the earlier results showing increased growth rate, verify the overexpression of *OXGR1* both at RNA and protein level, and localize OXGR1 expression. Additionally, it was studied whether varying concentrations of substrates relevant to the OXGR1 pathway have effect on the growth of OXGR1-overexpressing prostate cancer cells.

LNCaP and PC-3 cell lines were chosen for the studies because they are both well characterized prostate adenocarcinoma cell lines. PC-3 cells are more advanced prostatic cancer cells that are insensitive to androgens, while LNCaP cells are sensitive to androgens.

6.1 Verification of *OXGR1* overexpression and cellular localization of OXGR1

RNA expression was measured with standard RT-qPCR methods, and the *OXGR1* mRNA quantity was normalized to TBP mRNA. A major overexpression of *OXGR1* is seen in all the transfected cell lines (Figure 4). This is expected as *OXGR1* was overexpressed under cytomegalovirus (CMV) promoter, which typically induces a very strong expression. The RT-qPCR experiments have been repeated, making it fairly certain that the expression vector is performing as expected and that overexpression has been achieved. Stable transfection is performed by integrating *OXGR1* expressing pcDNA3.1 plasmids (Invitrogen) into cells'

genomes, so the differences between the RNA expressions of transfected lines could be due to differences in number of integrated plasmids.

Non-transfected cells and cells transfected with empty expression vector, both PC-3 and LNCaP, show minor *OXGR1* expression levels (table 2). This is expected, as Human Protein Atlas shows minor *OXGR1* expression in RNA sequencing of the normal prostate tissue samples (Fagerberg et al. 2014). siRNA silencing of *OXGR1* expression in LNCaP OXGR1_2 was performed to verify that the band seen in western blot experiments (Figure 9) with OXGR1 antibody is indeed OXGR1. In RT-qPCR measurements it is seen that anti *OXGR1* siRNA greatly reduces the RNA levels of *OXGR1*, around 90% (Figure 5). Yet, the siRNA treated sample still has *OXGR1* expression levels much higher than the controls not transfected with *OXGR1*.

The verification of OXGR1 overexpression at protein level proved to be challenging. In western blot experiment, anti-OXGR1 antibody produces a band of similar intensity in PC-3 cells with stable *OXGR1* transfection and in non- and control transfected cells (Figure 6). Results are the same with transiently transfected PC-3 cells and non-transfected control (Figure 7). With LNCaP cells, the bands produced with anti-OXGR1 antibody seem to be significantly more intense in the cells with stable *OXGR1* transfections than in the non- and control transfected cells (Figure 8). There is only a very minor difference in anti-OXGR1 band intensity between anti-*OXGR1* siRNA-treated LNCaP OXGR1_2 and non-targeting siRNA-treated LNCaP OXGR1_2 (Figure 9). This is somewhat unexpected, as 90% reduction in mRNA levels would be expected to translate to a detectable difference in protein level. It could therefore be considered an indication of non-specific antibody binding.

The clear bands seen in western blot experiments when probed with anti-OXGR1 antibody seem to have molecular weight of 45 kilodaltons or more (Figures 6-9), while the predicted size of OXGR1 based on amino acid composition is 38 kilodaltons. In addition, material from Abcam shows bands around 38 kilodaltons in size when various non-prostate tissue samples are probed with anti-OXGR1 antibody. In the western blot with stably transfected LNCaP cells two bands are seen, where the lighter one corresponds to the band seen in the western blot with stably transfected PC-3 cells, and the heavier one is not seen in PC-3 cells (Figures 6 and 8). These bands could be the result of differing post translational modifications of OXGR1, taking place only in LNCaP cells. Because of the protein denaturing during SDS-PAGE, different protein folds should not result in different bands in western blot.

Discrepancies in band size and the fact that substantial *OXGR1* overexpression at mRNA level doesn't seem to translate to high expression at protein level raise the question

whether the antibody is binding properly. The difference between predicted size and the band size in western blot experiments could be explained through post-translational modifications. For example, SUMOylation would cause a similar shift in band size. Regulation on the level of OXGR1 translation and degradation could keep the protein levels low despite the overexpression at RNA level. Formation of band due to non-specific binding of the secondary antibody was ruled out by probing PC-3 cells with secondary antibody only (Figure 7). It is still possible that the band seen in western blot experiments is due to non-specific binding of the anti-OXGR1 antibody, but this is unlikely since the experiments suggest a difference in protein expression level of the *OXGR1* transfected LNCaP cells.

One way to verify the presence of OXGR1 and to possibly gain insight on why the protein concentration is low or non-existent despite high overexpression at mRNA level, would be determination of protein half-life by cycloheximide chase analysis. In it, a flagged OXGR1 would be transfected to PC-3 cells, cycloheximide would be used to inhibit further protein synthesis, and protein degradation rate would be analyzed by western blots with anti-flag antibodies at chosen time points.

The results of the anti-OXGR1 immunostaining seem to be in line with the western blot results. OXGR1 expression was detected in roughly 20 to 30 percent of PC-3 cells in the population regardless whether the cells had been transfected with *OXGR1* or not (Figures 10 and 11). All cells with stable *OXGR1* transfection should be capable to express *OXGR1,* as the selection pressure produced with aminoglycoside antibiotic Geneticin® should eliminate cells with no expression vector. Since OXGR1 has a function in acid-base balance through regulation of bicarbonate secretion and inflammatory signaling through LTD4, expression of OXGR1 could be individual cell's response to these stresses (Kanaoka et al. 2013; Tokonami et al. 2013). This could explain the expression in only part of the cell population at a given time. OXGR1 has shown to be a G protein–coupled receptor (Wittenberger et al. 2002), so its localization mainly to the cell membrane is to be expected. Earlier study confirms this finding and shows that OXGR1 is internalized when bound to a ligand (He et al. 2004). Put together, the data from western blot and immunostaining experiments suggests that PC-3 cells natively express OXGR1 at modest levels and that its overexpression is kept in check by translational, post-translational and degradation regulation. Immunostaining of LNCaP cells could not be performed due to time constraints.

6.2 OXGR1 effects to cell proliferation

PC-3 cells with transient *OXGR1* transfection showed a minor increase in growth rate compared to cells transfected with empty vector (Figure 12). Further growth rate experiments were performed with cells with stable *OXGR1* transfections and non- and control-transfected PC-3 cells. Experiments were carried out in media containing different concentrations of key metabolic agents: glucose, glutamine, and AKG, along with regular media. Glucose is included as it is an important metabolite in aerobic and anaerobic metabolism and because tumors often utilize increased glucose uptake and increased utilization of glucose in biosynthetic processes (Anastasiou 2017). Glucose levels used ranged from fasting blood sugar (1 g/l) to standard for cells with high energy demand (4.5 g/l). Glutamine is the main link between the anabolic and catabolic amino acid metabolism and energy metabolism. It is also linked to redox state regulation through glutaminolysis to glutathione, which is a potent antioxidant. This pathway is typically over activated in prostate cancer due to elevated *MYC* oncogene activity (Fleming et al. 1986; Gao et al. 2009). Glutamine is also converted to AKG through glutamate in mitochondria. The glutamine levels used in this study ranged from starvation level (0 mM) to standard for cells with high energy demand (4 mM). AKG was chosen mainly as it is ligand for the OXGR1 (He et al. 2004). It also is an important intermediate in energy metabolism and anabolic and catabolic reactions. AKG levels used were starvation (0 µM), regular plasma level (25 µM), level required to activate OXGR1 (75 µM) and excess level (1 mM) (He et al. 2004). While there is no direct experimental evidence, it can be hypothesized that OXGR1 activation through binding to AKG is involved in energy metabolism and detecting surrounding metabolic states.

Varying glucose and glutamine levels don't seem to have much impact to growth rate of PC-3 cells, independent of *OXGR1* transfection status (Figure 13). Exception to this is the experiment with no glutamine in which the growth of all cell lines ceases. Most reliable data can be acquired from day 4 or 5 of the experiments, when the cells still have space to grow. Considering all experiments, non-transfected PC-3 cells seem to have the fastest growth rate, while *OXGR1* transfected cells seem to have the slowest growth rate (Figure 13). This may be explained by the burden the transfection imposes to cells, in particular to the ones producing excessive amounts of *OXGR1* mRNA. The experiment with varying AKG concentrations gave similar results, with no clear difference in growth between different cell lines (Figure 14). Because there is no difference in OXGR1 levels based on western blot, or even no OXGR1 present if the band is due to non-specific binding of the antibody, the results are expected.

Comparison is made difficult by high standard deviations between replicates. This is somewhat expected in this kind of experimental setup. Even seeding of the cells to the wells takes a lot of practice, and minor differences in the cell confluence in the beginning can lead to big deviations. There were also problems with the autofocus function of the microscope equipment, which may have caused part of the deviation. Some of the deviation could be eliminated by better equipment, more repeats of the experiment, and increasing the number of replicas in a way that outliers could be disregarded.

Growth rate experiment with varying AKG concentrations was repeated using LNCaP cell lines (Figure 15). In this experiment, there seems to be a modest increase in the growth rate of *OXGR1* transfected cell lines compared to the non- and control-transfected cell lines when AKG is present in the medium. Comparison is again made difficult by high standard deviations between replicas. Since there is a possible overexpression of OXGR1 at protein level in LNCaP cells, the minor increase in *OXGR1* expressing cells growth rate could indeed be caused by OXGR1. It is, however, also possible that the biological significance of OXGR1 overexpression, if any, lies in other aspects of cancer promotion than growth, for example immune and apoptosis evasion or tissue invasion.

6.3 Future directions

The results of the experiments with LNCaP cell lines are promising and they should be the focus of further studies. There is still concern that OXGR1 expression at protein level is not reliably verified with the antibody used in western blot experiments. The validation of antibody can be achieved by a combination of following methods: performing the immunocytochemistry experiment with LNCaP cells, repeating western blot experiments with other OXGR1 antibodies, examining cell lines or tissues of known *OXGR1* expression level and finally by producing an *OXGR1* knockout PC-3 cell line. Possible post-translational modifications of OXGR1 could be studied with different chromatography methods and mass spectrometry.

If the overexpression of OXGR1 in LNCaP cell lines can be confirmed, its biological effects must be put under further scrutiny. Cell invasion and migration studies are a logical next step, since they are relatively simple and affordable to perform for example using commercially available invasion chambers. By considering what is already known about the biological function of OXGR1, it is possible to make educated guesses about its function in cancer. One of the known functions of OXGR1 is to cause secretion of bicarbonate in kidneys in response to environment becoming more acidic (Tokonami et al. 2013). This causes better buffering and neutralization of extracellular pH, which is somewhat counterintuitive when considering cancer

promotion. Acidic microenvironment is considered a hallmark of cancer and it can promote cancer through three major mechanisms: by destruction of adjacent normal cell populations, by acid-induced degradation of extracellular matrix, and by angiogenesis promotion (Gatenby and Gillies 2004). OXGR1 is also shown to be a receptor for LTE_4 (Kanaoka et al. 2013), a cysteinyl leukotriene involved in pulmonary inflammation and vascular permeability (Maekawa et al. 2008). It has also been shown that it is indeed OXGR1 that mediates the increased vascular permeability response to LTE_4 (Kanaoka et al. 2013). Increased vascular permeability is a known cancer promoting factor that can facilitate metastasis and modulate immune response (Farnsworth 2014). One possible way to study this line of thought further is to overexpress *OXGR1* in a mouse model that has increased likelihood of developing prostate cancer, and compare cancer incidence, immune response markers, and histopathology of the tumors.

7 CONCLUSIONS

Main goals of the study were to confirm OXGR1 overexpression in transfected prostate cancer cell lines PC3 and LNCaP, to corroborate earlier experiments showing increased growth rate of cells transfected with *OXGR1* and to study the effect of key substrates to growth of transfected cells.

OXGR1 overexpression could not be confirmed in PC-3 cells. Western blot experiments showed a band of similar intensity in the *OXGR1* transfected lines and in the control cell lines. Correspondingly the immunocytochemistry showed similar expression patterns in transfected and control cell lines. There remains some concern whether the antibody performed properly, since the band detected was ~10 kilodaltons larger than predicted. Growth rate experiment with transiently transfected PC-3 cells showed a minor increase in growth rate in transfected cells compared to control cells. This result could not be confirmed with stable *OXGR1* expressing cell lines. Adjustment of concentration of glucose, glutamine and AKG in growth medium didn't have an effect to growth rate of *OXGR1* transfected PC-3 cells in comparison with control cells.

Western blot experiments with *OXGR1* transfected LNCaP cells showed a band of same molecular weight as in PC-3 cells that was missing from the control LNCaP cells. Growth rate experiments show a minor increase in growth rate of *OXGR1* transfected LNCaP cells, in comparison to control LNCaP cells, when AKG is present in the growth medium.

Since a clear difference is seen in intensity of western blot bands produced with OXGR1 antibody between transfected and control LNCaP cells, it is likely that the antibody is working properly and the difference to predicted band size is due to post-translational modifications to OXGR1. This result must be validated in future studies. This would mean that the PC-3 cells have basal expression of OXGR1 and that the excess mRNA is either not translated to protein, or the excess protein is rapidly degraded. As overexpression of OXGR1 is achieved in LNCaP cells, the stable expressing cell lines can be used in further studies to elucidate the possible role of OXGR1 in prostate cancer promotion.

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